THE ROLE OF BILE ACID RECEPTOR FXR AND HDL IN INTESTINAL INFLAMMATION

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Wenn jemand sucht, dann geschieht es leicht, daß sein Auge nur noch das Ding sieht, das er sucht, daß er nichts zu finden, nichts in sich einzulassen vermag, weil er nur an das Gesuchte denkt, weil er ein Ziel hat, weil er von Ziel besessen ist. Finden aber heißt: frei sein, offen stehen, kein Ziel haben.

Hermann Hesse

To my parents Rajan John and Martha Attinkara

sister Renjini, brother Robin, appachen John Attinkara

and companion Raphael Gerster

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SUMMARY

Inflammatory bowel disease (IBD) characterizes chronic gastrointestinal inflammation manifested by abdominal pain, diarrhea, malabsorbtion and bleeding, of which two phenotypes, Crohn's disease (CD) and ulcerative colitis (UC) have been described [1-3]. It affects about 1.4 million people in the USA and 2.2 million people in Europe [4, 5].

The nuclear receptor farnesoid X receptor senses intracellular bile acid levels. In response to elevated levels of intracellular bile acids, activated FXR induces gene expression of bile acid efflux transporters whereas the expression of bile acid uptake transporters are down regulated, thus protecting against bile acid toxicity in the liver and intestine. FXR regulates several genes that can protect against intestinal inflammation and bacterial overgrowth. FXR-deficient mice have increased ileal concentrations of gut bacteria and exhibit defects in the integrity of the intestinal epithelial barrier. Furthermore, in rodent models of colitis and in CD patients, it has been reported that reduced expression of FXR is associated with colon inflammation [6]. The fact that FXR thus appears to play a role in the protection of the integrity of the intestinal epithelial barrier and its inverse correlation with the level of intestinal inflammation suggest a potential connection between FXR and the molecular pathogenesis of IBD.

In the first chapter of this thesis, we have investigated five FXR single nucleotide polymorphisms - two common SNPs and three rare variants - which have been previously studied in the context of human disease, in a well sized IBD vs non-IBD cohort, and report that two of these genetic variants are associated with IBD.

NF-κB activity is upregulated in patients with IBD, characterized by high circulatory levels of cytokines especially tumor necrosis factor (TNF). Low plasma concentration of high density lipoprotein (HDL) cholesterol is a marker and considered as a risk factor for coronary heart disease, diabetes mellitus, and several cancers including colorectal cancers [7-9].

The intestine plays a crucial role in HDL metabolism. In addition, the classical anti-atherogenic function of HDL, the mediation of reverse transport of excess cholesterol from macrophages of the arterial wall to the liver is completed by biliary excretion of cholesterol into the intestine. Intestinal dysfunction and inflammation leads to decrease in serum levels of HDL cholesterol and ApoA1 [10-13]. Consistent with this, clinical studies show

significantly lower HDL-cholesterol levels in patients with active IBD [14]. Traditionally, the low HDL cholesterol in IBD patients is interpreted as the consequence of IBD. The association of low HDL-cholesterol with IBD together with the many anti-inflammatory properties of HDL however raises the question of whether HDL and its protein and lipid components have any causal impact on intestinal inflammation in IBD patients.

In the second chapter of the thesis, we approached this question and investigated the effect of HDL and ApoA1 on intestinal inflammation both *in vitro* and *in vivo*.

Autophagy, a catabolic process involving intracellular degradation of organelles and proteins, has been shown to be compromised in many human diseases including IBD. Autophagy malfunction has been attributed to defective clearance of pathogens and dysfunction of paneth and goblet cells, which are relevant for pathogenesis of IBD. In the third chapter of the thesis, we put forward autophagy as a mechanism by which HDL suppress intestinal inflammation.

ZUSAMMENFASSUNG

Chronisch entzündliche Darmerkrankungen (IBD), bestehend aus Morbus Crohn (CD) und Colitis ulcerosa, (UC) charakterisieren chronisch gastrointestinale Entzündungen, welche durch Bauchschmerzen, Durchfall, Malabsorption und Blutungen manifestiert werden [1-3]. Es betrifft etwa 2,2 Millionen Menschen in Europa und 1,4 Millionen Menschen in den USA [4, 5].

Der Kernrezeptor Farnesoid X-Rezeptor (FXR) stellt intrazelluläre Gallensäure fest. In Reaktion auf erhöhten intrazellulären Gallensäuren, induzieren aktivierte FXR Genexpression von Gallensäure Effluxtransporter, wohingegen die Expression von Gallensäure-Aufnahme Transportern herunterreguliert sind, und somit Schutz gegen Gallensäure Toxizität in der Leber und Darm bieten. FXR regelt mehrere Gene, die gegen Darmentzündungen und bakteriellen Überwucherung schützen können. Fxr-defiziente Mäuse haben erhöhte ileale Konzentrationen von Darmbakterien und weisen Defekte in der Integrität und Funktion des intestinalen Epithelbarriere auf. Weiterhin wurde berichtet, dass eine reduzierte Expression von FXR mit Darmentzündung in Nagermodellen von Colitis und im CD-Patienten assoziiert wird [6]. Die Tatsache, dass FXR somit eine Rolle beim Schutz der Integrität der intestinalen Epithelbarriere und seine inverse Korrelation mit dem Niveau der intestinalen Entzündungen spielt, scheint auf eine potentielle Verbindung zwischen FXR und der molekularen Pathogenese der IBD zu deuten. Zwei gemeinsame SNPs und drei seltene Varianten wurden in einem IBD vs nicht-IBD-Kohorte untersucht.

Im ersten Kapitel dieser Arbeit haben wir fünf FXR Single Nucleotide Polymorphismen untersucht und berichten, dass zwei dieser genetischen Varianten mit IBD verbunden sind.

NF-kB-Aktivität wird bei Patienten mit IBD durch hohe Konzentration von insbesondere Zytokine Tumornekrosefaktor (TNF) hochreguliert. Eine niedrige Plasmakonzentration von Lipoprotein mit hoher Dichte (HDL) ist ein Risikofaktor für koronare Herzkrankheit, Diabetes mellitus und einige Krebsarten wie Darmkrebs [7-9,10-13].

Der Darm spielt eine wichtige Rolle in HDL Stoffwechsel. In der klassischen antiatherogenen Funktion der HDL, wird überschüssiger Cholesterin aus Makrophagen der Arterienwand in die Leber durch HDL zurück transportiert und endet mit biliären Ausscheidung von Cholesterin in den Darm.

Disfunktionen und Entzündungen im Darm führen zu niedrigen Serumspiegel von HDL-Cholesterin und ApoA1 [14-17,18-21]. Im Einklang mit dieser zeigen klinische Studien deutlich niedrigere HDL-Cholesterinspiegel bei Patienten mit aktiver IBD [22]. Traditionell wird das niedrige HDL-Cholesterin bei IBD-Patienten als Folge der chronischen Entzündung interpretiert. Der Verband der niedrigen HDL-Cholesterin mit IBD wirft zusammen mit den vielen anti-inflammatorische Eigenschaften von HDL allerdings die Frage auf, ob HDL und sein Protein/Lipid-Komponente einen Einfluss auf die Darmentzündung bei IBD-Patienten haben. Im zweiten Kapitel der Dissertation näherten wir uns dieser Frage und untersuchten die Wirkung von HDL und Apo-A1 auf Darmentzündung, sowohl *in vitro* als auch *in vivo*.

Autophagie ist ein intrazellulärer Abbauprozess, das in vielen menschlichen Krankheiten einschließlich IBD beeinträchtigt werden. Autophagy Fehlfunktion führt zu mangelhafter Clearance von Pathogenen und Dysfunktion der Paneth- und Becherzellen, die relevant für die Pathogenese der IBD sind. Im dritten Kapitel der Arbeit schlagen wir Autophagie als eine mögliche Mechanismus vor, durch der HDL die Darmentzündung unterdrückt.

1 INTRODUCTION

1.1 Physiology of intestine

The small intestine and colon primarily form the gut whose primary function is digestion and absorption of nutrients. The small intestine is structurally divided into the duodenum, jejunum and the ileum. Epithelial protrusions called villi increases the surface area of the small intestine. The intestinal mucosa is constantly exposed to dietary antigen and commensal bacteria. The gut therefore has to keep a state of balance on one hand preventing overwhelming immune responses whereas at the same time maintaining mucosal integrity.

A monolayer of intestinal epithelium build a physical barrier to foreign antigen and influence immune cell proliferation and differentiation by producing a myriad of signaling molecules. The intestinal epithelial monolayer include columnar epithelial cells with absorptive function, goblet cells that secrete mucous, Paneth cells that secrete antimicrobial defensins as well as lysozymes and hormone secreting enteroendocrine cells, forming a physiological barrier against commensal and invasive pathogens which is equally important as its role in absorption of water, salts and nutrients (Fig 1). Columnar epithelial cells also known as enterocytes are polarized with an apical brush border that is in contact with the luminal contents and a basolateral membrane adjacent to stroma. Each enterocyte is connected by intercellular junctions (Tight junction, adheren junction and desmosomes) which contribute to the barrier function. A coat of glycoproteins (mucin) occupies surface receptors of enterocytes, limiting the attachment of microbes from the lumen. Beneath the mucin layer lies the glycocalyx layer with adsorbed enzymes on the negatively charged microvilli, responsible for digestion. In addition, lymphoid aggregates called Peyers patches are distributed in the epithelia. These are overlaid by Microfold (M) cells that deliver antigen to lymphoid follicles of Peyer’s patches. The lamina propria with antigen presenting cells (APCs) such as dendritic cells and macrophages lies beneath the epithelia separated by a basal membrane [15]. The intestinal epithelium is renewed every 4-5 days by proliferative crypts of Lieberkühn which contain intestinal stem cells. Disruption of the barrier can lead to an uncontrolled inflammation triggered by microbes as

seen in inflammatory bowel disease (IBD) [16, 17]. The epithelia also act as antigen presenting cells, capable of internalizing (via endocytosis or pinocytosis), processing and presenting foreign antigen to dendritic cells in the lamina propria.

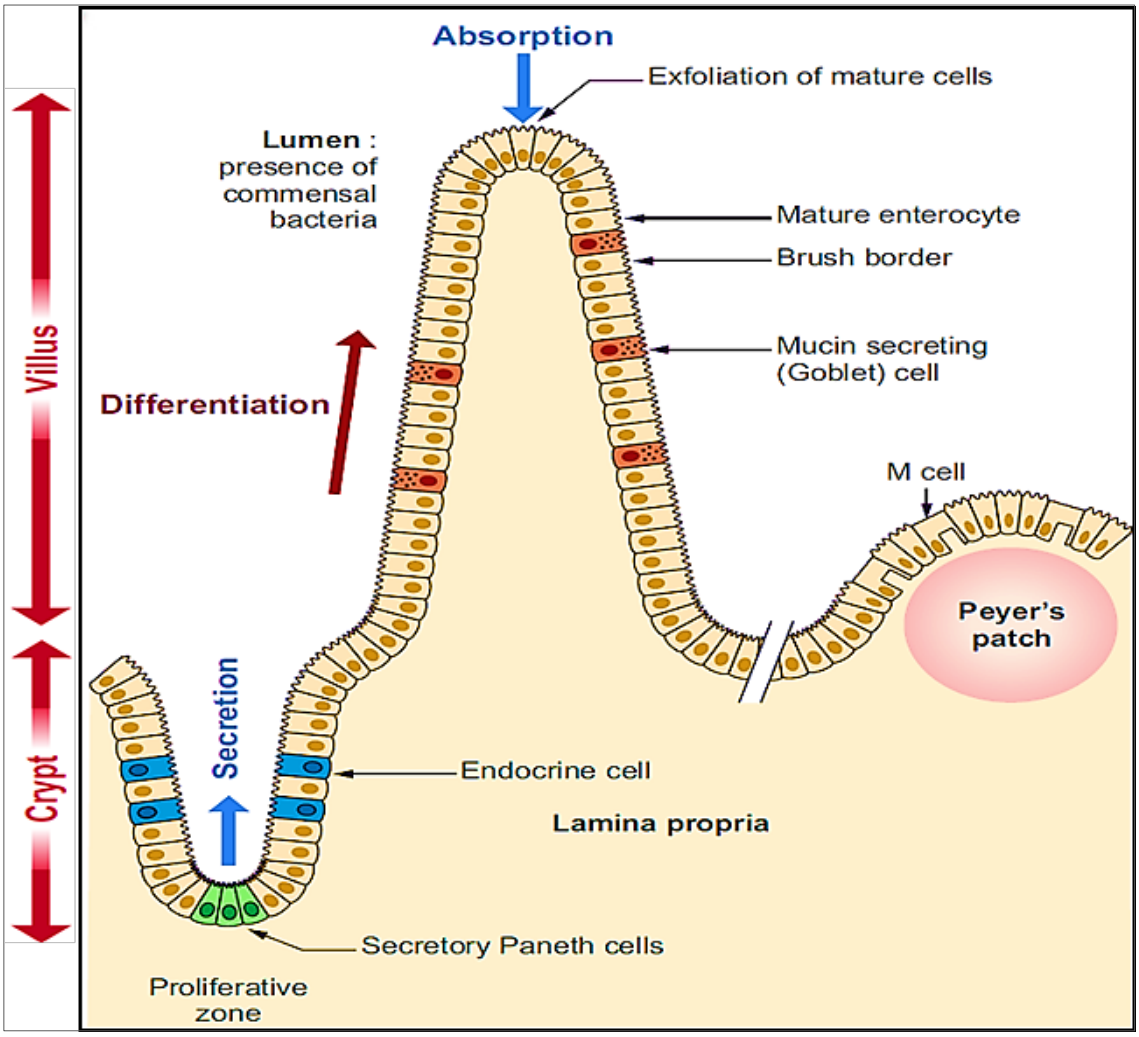


Figure 1: Intestinal Epithelium

(adapted from [18])

Intestinal epithelium is involved in immune cell regulation and antigen processing despite building a physical barrier against commensal bacteria and foreign antigen. The epithelia is folded into crypts and villous protrusions. Intestinal epithelial cell differentiation takes place in the crypt. Four types of intestinal epithelial cells are derived from pluripotent stem cells of the crypt. The goblet, enteroendocrine and absorptive cells, which migrate to the tip of villus where as the fourth lineage, paneth cells remains within the crypt [18].

1.2 Immune functions of intestinal mucosa

The intestine forms the largest part of immune system with a surface area of 400 m^2 being exposed to enormous antigenic burden of dietary or commensal origin. Atleast 10^{13} to 10^{14} commensal bacteria cohabit the gastrointestinal tracts carrying out essential functions such as digestion, nutrient supply (especially vitamin B and folates) and fine tuning mucosal immune system [19, 20] . Despite the extreme antigenic burden, the epithelia maintain a passive mucosal inflammatory response which is achieved by limiting initial immune responses via secretion of anti-inflammatory cytokines and recruiting immune regulatory cells. The gut associated lymphoid tissue, lamina propria cells and enterocytes actively play a role in maintaining passive unresponsiveness to antigens. The epithelium produces antimicrobial proteins such as lysozymes, defensins, cathelicidins, RNases and C-type lectins to resist microbial invasion of the tissue. The most abundant antibody isotype produced in the body, secretory Immunoglobulin A (IgA), is transcytosed to apical and basolateral surface where they bind to polymeric Ig receptor providing mucosal protection and contribute to barrier function of the epithelium. The epithelial monolayer forms a thin physical barrier preventing penetration of pathogens from the intestinal lumen [21, 22] . Intraepithelial lymphocytes which express αβ (with CD4 or CD8 coreceptors) or γδ (with CD8 coreceptor) T-cell receptors, monitor stressed or damaged intestinal epithelial cells [23, 24]. The intestinal epithelia express immune receptors such as Toll like receptors (TLR), Nod like receptor (NLR) and C-type lectin receptor (CLR) which recognize and bind pathogen associate molecular pattern (PAMP) expressed on microbial surface and upregulate antimicrobial factors as well as secretion of cytokines and chemokines via activation of NF-κB pathway [25, 26]. Mutations in nucleotide oligomerization domain-2 (NOD2/CARD15) also known as IBD1 which recognizes muramyl di peptide (MDP), is strongly linked to IBD with a strong predisposition to CD [27]. Variants in TLR4 gene which recognizes LPS has also been implicated [28]. The gut associated lymphoid tissue (GALT) is scattered throughout the epithelia, lamina propria Peyer's patches and mesenteric lymph nodes (MLN) and is rich in B and T lymphocytes, [29]. The Peyer's patches are lymphoid aggregates in the submucosa, consisting of collection of B cell

follicles and T cell areas. The MLNs which are the largest lymph nodes in the body lies between layers of mesentry, stretching across jejunum and ileum.

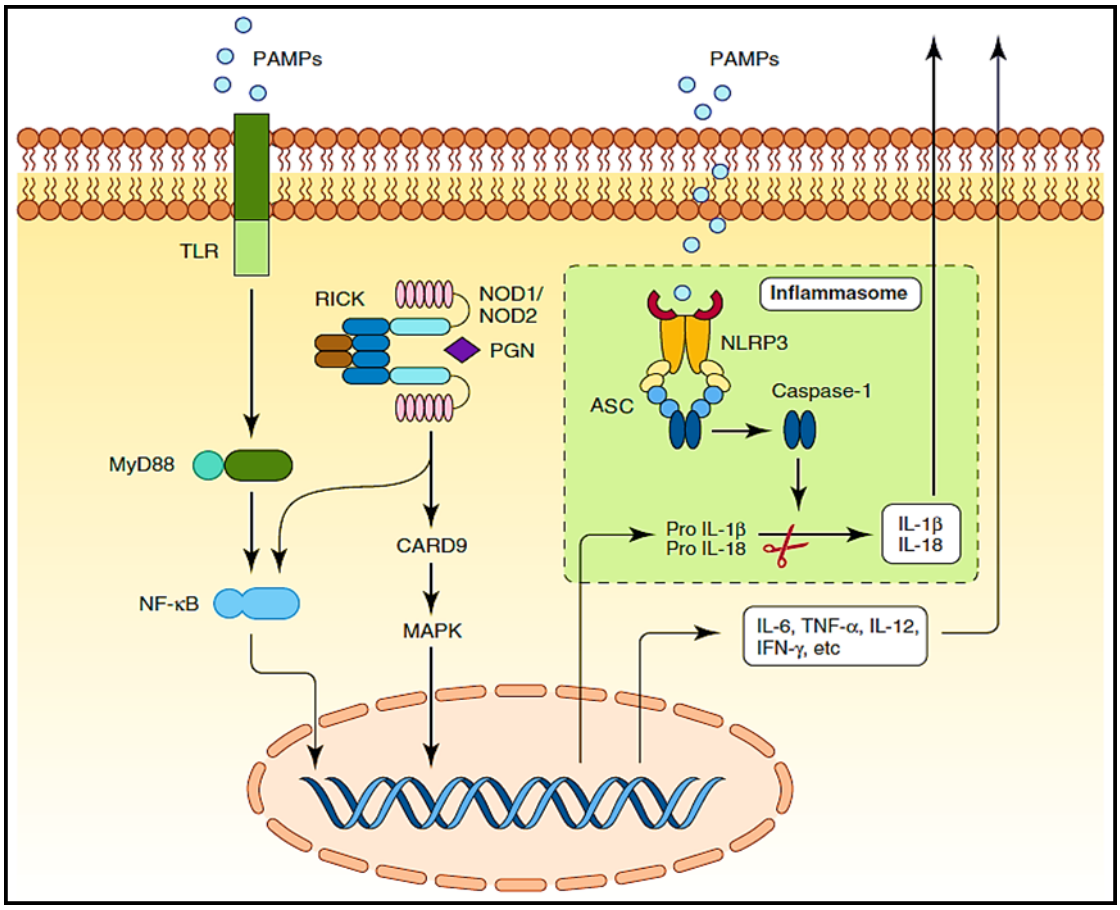


Figure 2: Intestine as an immune organ

(adapted from [30])

Intestinal epithelial cells sense PAMPs through TLR and NLR pattern recognition receptors, which activates transcription factor NF-κB via MyD88. NLR family members NOD1 and NOD2 senses peptidoglycans (PGN), a component of bacterial cell wall inducing recruitment of RICK and adapter protein CARD9, further activating NF-κB and MAP kinases which induces the expression of proinflammatory cytokines. NOD like receptor protein 3 (Nlrp3) senses patterns of microbial and non microbial origin and forms a multiprotein complex with apoptosis associated speck like protein (ASC) and CARD domain of caspase-1 called the inflammasome which plays a crucial role in the inflammatory process by activating caspase-1 and mediates production of pro-inflammatory cytokines IL-1β and IL-18 [30].

1.3 Pathogenesis and genetics of inflammatory bowel disease (IBD)

Inflammatory bowel diseases (IBD) characterizes a chronic disease manifested by recurrent inflammation of intestinal mucosa that results from aberrant regulation of the mucosal immune system resulting in excessive production of inflammatory cytokines, in genetically susceptible individuals. The etiology of IBD involves a complex interaction of genetic, environmental, and immunomodulatory factors. Two major forms of chronic mucosal inflammation have been defined. In Crohn's disease (CD), any part of the gastrointestinal tract may be affected, although the most frequent site of inflammation is the terminal ileum, whereas in ulcerative colitis (UC) inflammation typically affects the mucosa of colon (Fig 3) [31].

In IBD, both UC and CD are characterized by a weakened gut barrier, with increased infiltration of gut bacteria. Recruitment of neutrophils and formation of crypt abscess following antigen infiltration is typical for UC, where as in CD, macrophages are recruited with formation of granulomas [32, 33]. Tissue damage occurs mainly due to heavy infiltration of CD4+ T lymphocytes which are recruited from blood stream via enhanced production of chemoattractants such as CCL25 which promotes migration of CC chemokine receptor 9 (CCR9)- expressing T-cells to the intestinal mucosa [34]. Cytokines such as IL-15 and IL-21 which trigger anti-apoptotic programs in CD4+ T cells are produced in excess in IBD which may contribute to diminished susceptibility of IBD associated T-lymphocytes to apoptosis, leading to their accumulation in the gut wall, resulting in chronic inflammation [35-37]. The pathogenesis of CD exhibit a predominant Th1 mediated response with increased levels of interferon-γ and TNF as well as Th17 cytokine IL17, UC by contrast is characterized by an exaggerated Th2 response with increased production of IL13 and IL17 [38, 39]. Polymorphisms of multiple genes in the IL23 pathway that is central to the function of Th17 cells have been associated with CD and UC [40-42]. For CD pathogenesis, a strong genetic component has been suggested by the concordance of 63.6% in monozygotic twins, but only of 3.6% in dizygotic twins. The concordance of monozygotic twins is lower (6%) in UC, indicating that genetic susceptibility may play a somewhat smaller role in this disease [43]. Prior studies have revealed more than 100 genes that are potentially associated with IBD [44, 45]. The region

on chromosome *16q11-12* named *IBD1* was identified in 1996, and the fine mapping of this region led to the identification of the *NOD2* (nucleotide-binding oligomerization domain 2)/*CARD15* (caspase activation recruitment domain 15) gene [46, 47] a member of a family of pattern recognition receptors (PRRs) that recognizes microbial components and modifies inflammatory responses to bacterial triggers, such as lipopolysaccharides (LPS), through the activation of NF-κB [48, 49]. Furthermore, genes that are essential in immunological cell-cell interactions and signalling, such as the tumour necrosis factor receptor 1 (*TNFR1*) [50], the interleukin-23 receptor (*IL23R*) [51], and other genes that are involved in immune response to bacteria, such as the toll-like receptor 4 (*TLR4*) [52, 53], have been proposed to be associated with IBD. In addition, regulatory genes, such as the protein tyrosine phosphatase N2 (*PTPN2*) [54, 55] and the anti-inflammatory nuclear receptor peroxisome proliferator-activated receptor-γ (*PPARγ*) [56], as well as genes encoding membrane transporters multidrug resistance gene 1 (*MDR1)* [57-59] and the organic cation transporter 1/2 (*OCTN1/2*) [57, 60] have been associated with the risk of chronic mucosal inflammation.

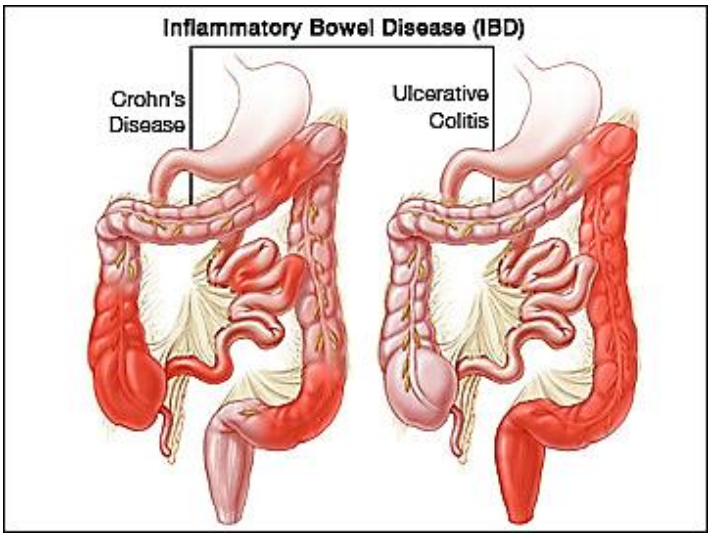


Figure 3: Phenotypes of inflammatory bowel disease
(adapted from http://iconsinmedicine.wordpress.com/2011/02/28/inflammatory-bowel-disease)

In UC, inflammation is confined to the colon and the inner lining of the intestinal wall is affected whereas in CD, the ileum is frequently inflamed. Both are characterized by diarrhea, weight loss, rectal bleeding, abdominal cramps, fever and pain.

1.4 NF-κB – a key regulator of intestinal inflammation

The transcription factor NF-κB plays a crucial role in initiating inflammatory cascades in response to extracellular stimuli such as microbial products, cytokines, reactive oxygen species, hypoxia and endoplasmic reticulum stress [61]. The intestinal tract is prone to inflammatory insult due to presence of endogenous microflora as well as food derived antigens. Activation of NF-κB is pivotal in preserving intestinal immune homeostasis. Five proteins, Rel A (P65), Rel B, c-Rel, P105 and P100 constitute the NF-κB family of transcription factors in mammals. These proteins form homo- or heterodimers to bind to κB sites in the promoter region of target genes. Rel A, Rel B and c-Rel contain a transactivation domain capable of activating κB containing promoters, whereas P105 and P100 have an inhibitory domain consisting of ankyrin repeats which has to be cleaved to active P50 and P52 respectively [62]. NF-κB dimers are retained inactive in the cytoplasm bound to an inhibitory protein IκB (inhibitor of κB). IKK (IκB kinase) complex which consists of two catalytically active kinases IKKα and IKKβ and a regulatory subunit IKKγ (NEMO) is activated by a wide range of stress signals such as TNF or LPS. IKK phosphorylates IκB at its serine residues and marks for K48 ubiquitination and degradation by proteasome, the released NF-κB dimer translocates to the nucleus and transcribes genes involved in inflammation such as TNF, IL-1β, IL-6, IL-8, ICAM, iNOS, (Fig 4) [63-65]. Two types of NF-κB signaling pathways have been described. The canonical pathway is induced by activation of TNFR1 (tumour necrosis factor receptor 1) by pro-inflammatory signals such as cytokines, PAMPs and DAMPs (danger associated molecular patterns), leading to binding of TRADD (TNF receptor associated death domain) which recruits TRAF2. TRAF2 associates with receptor interacting protein kinase1 (RIPK1) and binds inhibitor of apoptosis (cIAPs), to activate IKK. IκB is phosphorylated by IKKβ and NEMO in the canonical pathway. In contrast, the non-canonical pathway is induced by cytokines such as CD40L and lymphotoxin-β (LT-β) leading to activation of NF-κB inducing kinase (NIK) which activates IKKα that further phosphorylates and processes P100 associated with RelB to generate the transcriptionally active P52-Rel B complex [64, 66, 67]. This pathway is important during B and T cell development. Post translational

modifications (phosphorylation, acetylation and prolyl isomerization) of NF-κB subunits have been shown to modulate their transcriptional activity [68] .

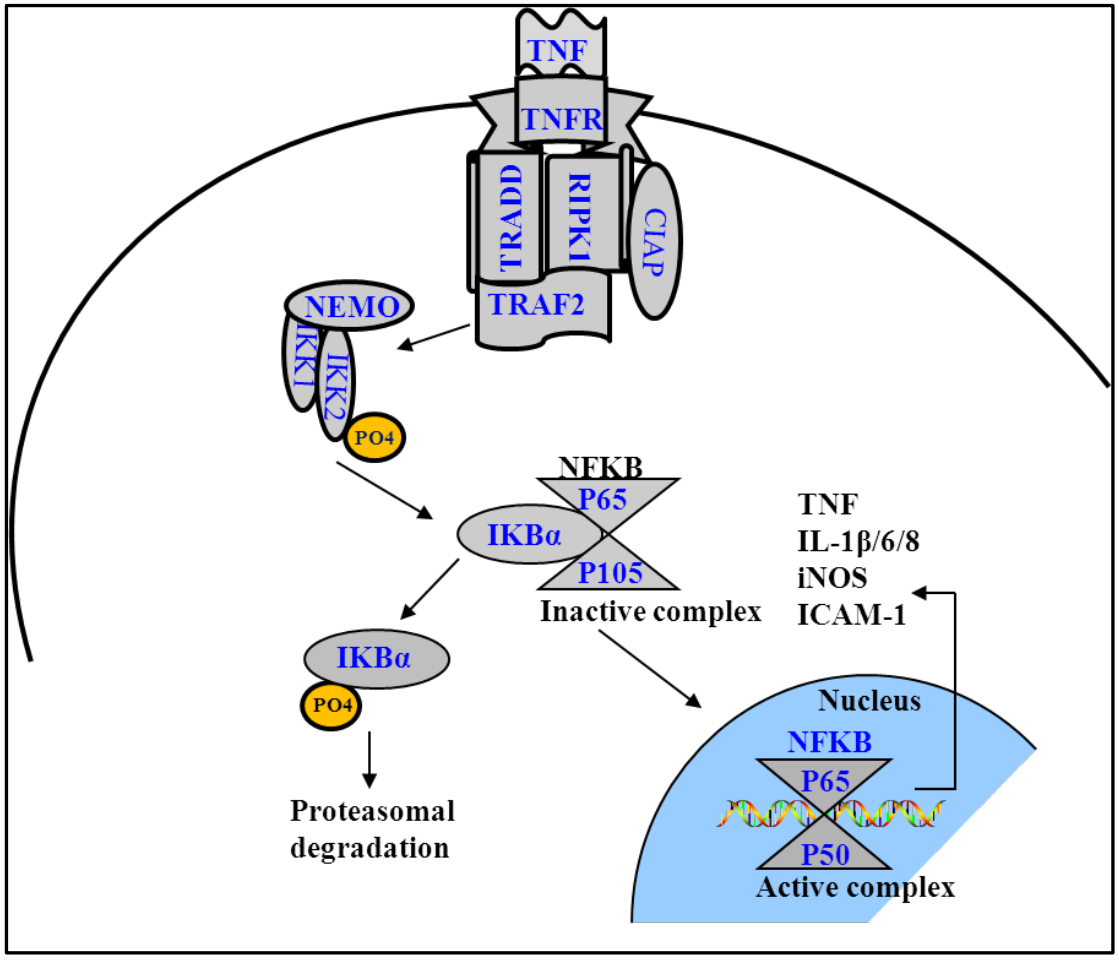


Figure 4: The canonical pathway of NF-κB activation

NF-κB is activated through IKK mediated phosphorylation followed by proteasomal degradation of inhibitory protein IκB, releasing NF-κB subunits, which translocate to the nucleus and induce target gene expression. In the canonical pathway, binding of ligands such as TNF to its receptor recruits adaptors TRAF2 and TRADD to the cytoplasmic domain of the receptor, which further interact with RIPK1 and cIAP activating the IKK complex.

1.5 TNF – the major cytokine initiating intestinal inflammation

TNF is a 26 kDa protein, which is secreted and expressed on the plasma membrane of activated macrophages and T –lymphocytes and is cleaved by matrix metalloproteinases to a soluble 17 kDa protein. Both forms of TNF exert their effect by binding as a trimer to cell membrane receptors TNFR-1 (55 kDa) or a TNFR-2 (75 kDa) [69-71]. Clathrin coated pits internalize the ligand receptor complex which fuse with lysosomes where TNF is degraded. Binding of TNF to TNFR-2 is not sufficient to attain cytotoxicity whereas binding to TNF-R1 mediates cell toxicity [72]. TNF exerts its effect by activating a number of proteins: transcription factors such as AP-1, NF-κB, protein kinases (erk-1, erk-2 and MAP2), phospholipases (PLA2, PLC, PLD and sphingomyelinase), mitochondrial proteins (manganese superoxide dismutase) and caspases [70, 73-75]. The biological functions of TNF include immunostimulation, resistance to infectious agents, resistance to tumours, sleep regulation and embryonic development [75-77]. Many cell types including macrophages, monocytes, lymphocytes, mast cells, leukocytes, astrocytes, microglial cells, keratinocytes, tumor cells and intestinal Paneth cells produce TNF. During inflammation, TNF is secreted by T helper 1 cells and act synergistically with IFNγ and IL17 to propagate inflammation. In contrast TNF downregulates T-cells and releases nitric oxide and prostaglandin from macrophages to regulate chronic TNF secretion [78-81]. mRNA expression of TNF is regulated at the level of mRNA stability and translation [82-84]. The AU-rich element (ARE) at the 3' un-translated region (UTR) of TNF confers mRNA instability and repression of TNF in unstimulated cells. Interaction between RNA binding protein Tris tetra proline (TTP) with 3'-UTR ARE of TNF modulates recruitment of exosomes for degradation of TNF, thus increasing the rate of TNF mRNA turnover under basal conditions [85-87].

TNF is a major mediator in the pathophysiology of inflammation especially in IBD. In animal models of colitis, TNF expression is significantly increased in the inflamed intestine and inhibition of TNF appears to reduce the disease severity [88-91]. Currenly three TNF blockers are in clinical use to treat IBD. Infliximab, a chimeric mouse human IgG1 monoclonal antibody to TNF, is able to neutralize circulating and membrane bound TNF as well as induce apoptosis of T cells and monocytes [92-99]. Adalimumab is a

recombinant human monoclonal antibody which block TNF interaction with its receptors and induce T cell and monocyte apoptosis like infliximab [100]. Certolizumab pegol is a pegylated monoclonal antibody which bind and neutralizes membrane associated and soluble human TNF [101]. Inhibitors of TNF have been shown to be relieve symptoms in chronic inflammatory diseases such as IBD, psoriasis, ankylosing spondylitis and psoriatic arthritis [102].

1.6 Nuclear receptor FXR and intestinal inflammation [103]

Nuclear receptors represent a large family of transcription factors that regulae numerous processes, including reproduction, development, and a wide range of metabolic pathways [104]. The ligand-dependent activation function at the carboxy-terminus of most nuclear receptors allows them to sense metabolic changes within cells, and in response carry out rapid transcriptional changes [105-107].

The farnesoid X receptor (FXR); gene symbol *NR1H4* (nuclear receptor subfamily 1, group H, member 4) is a nuclear receptor that functions as the main sensor of intracellular bile acid levels [108-110]. The human *NR1H4* gene, composed of 11 exons and 10 introns is located on chromosome 12 [111]. The translation initiation codon of the *NR1H4* gene lies at the 3′ end of exon 3, whereas exons 1 and 2, together with the 5′ region of exon 3, contain the 5′ non-translated region (5’-UTR).

Multiple FXR isoforms can be generated via alternative promoter usage and alternative splicing, and these isoforms may have differential transactivation abilities on specific target promoters [112]. FXR typically acts by binding to FXR response elements within the target promoters as heterodimers with another member of the nuclear receptor family, retinoid X receptor-α (RXRα) [113]. In response to elevated levels of intracellular bile acids, activated FXR is well known to induce protective gene expression circuits against bile acid toxicity in the liver and intestine [114]. Expression of bile acid efflux systems in ileocytes (organic solute transporter α/β; OSTα/β) [115, 116] and hepatocytes (bile salt export pump; BSEP) [117-119] is upregulated by bile acid-activated FXR, while the expression of the respective bile acid uptake systems apical sodium-dependent bile acid

transporter (ASBT) [120] and Na^+-taurocholate co-transporting polypeptide (NTCP) [121, 122] is suppressed by it. Transcription of the three major genes coding for bile acid synthesizing enzymes, namely cholesterol-7α-hydroxylase (CYP7A1), sterol-12α-hydroxylase (CYB8B1), and sterol-27-hydroxylase (CYP27A1) are repressed by FXR [123, 124]. Thus, elevated levels of bile acids can suppress their own *de novo* production through a negative feedback loop involving FXR (Fig 5). In addition, FXR regulates several genes that can protect against intestinal inflammation and bacterial overgrowth [6, 125, 126]. Fxr-deficient mice have increased ileal concentrations of gut bacteria and exhibit defects in the integrity of the intestinal epithelial barrier. In agreement with this, the products of a number of genes that are regulated by Fxr in the ileum, including angiogenin (*Ang1*), inducible nitric oxide synthase (*iNos*), and interleukin-18 (*Il-18*), are known to have antimicrobial actions [126]. Furthermore, it has been reported that reduced expression of FXR is associated with colon inflammation in rodent models of colitis and in CD patients [6].

Recently, FXR activation was shown to decrease NF-κB-mediated immune responses and intestinal permeability in mouse models of colitis [127]. It was subsequently shown that intestinal inflammation reduces FXR activation as well as the expression of FXR target genes such as intestinal bile acid-binding protein (*IBABP*) and fibroblast growth factor 15/19 (*FGF15/19*) [128]. In agreement with this, it has been proposed that FXR may contribute to the resistance of both human and mouse gastric epithelial cells against inflammation-induced injury [129].

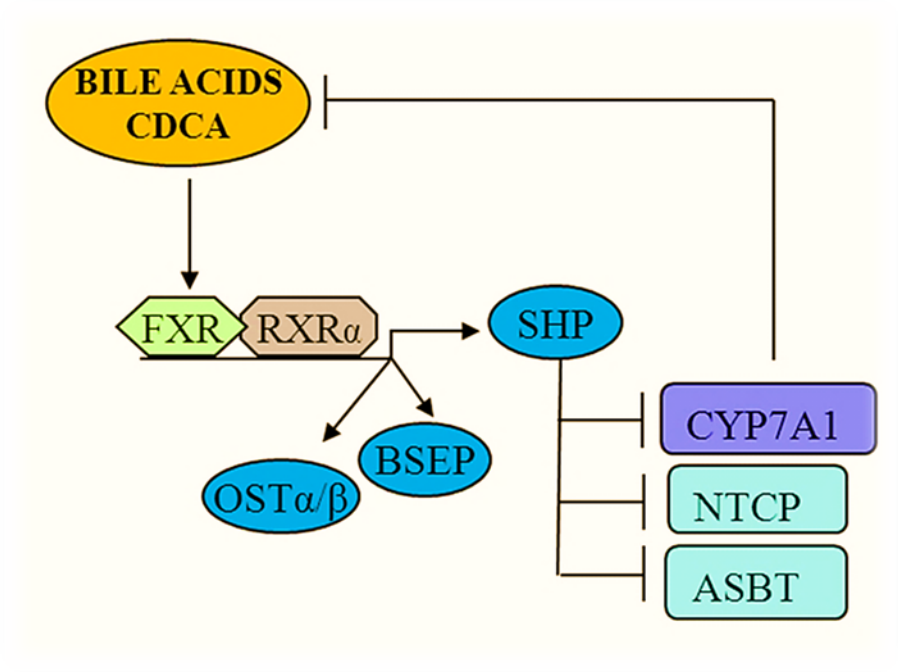


Figure 5: Regulation of bile acid homeostasis

CDCA, the major component of bile acids, is a potent ligand of FXR, inducing heterodimerization of FXR with RXRα leading to induction of bile acid efflux transporters OSTα/β and BSEP. Also a repressor protein SHP is induced which inhibits the expression of CYP7A1 thus suppressing bile acid synthesis. Also bile acid uptake transporters such as NTCP and ASBT is repressed via SHP thus protecting hepatocytes against bile acid overload and toxicity.

1.7 High density lipoprotein

High density lipoproteins are a class of spheroidal plasma lipoproteins with a density of 1.063 to 1.21 g/mL comprising of apolipoproteins (ApoA1 ApoAII and ApoCIII), phospholipids and a core of cholesteryl esters [130]. They are the smallest and densest of lipoprotein particles with highest proportion of protein to cholesterol. Five subfractions of HDL have been defined, varying in size (8-11 nm in diameter) and density. HDL has been shown to promote cholesterol efflux from artery walls and peripheral tissues, transporting it back to the liver in a process called reverse cholesterol transport (RCT). Low levels of HDL cholesterol (HDL-C) in plasma is inversely related to the risk of cardiovascular

disease [131, 132]. An increment of 1mg/dL in HDL-C has been shown to reduce mortality from cardiovascular disease by 3-4% [133].

ApoA1, the major protein of HDL is secreted by liver and intestine which form nascent HDL after acquiring phospholipids and free cholesterol via interaction with the ATP-binding cassette A1 (ABCA1) transporter [134]. Mature HDL is generated by Lecithin cholesterol acyltransferase (LCAT) which esterifies free cholesterol to cholesteryl esters. This process increases the cholesterol uptake capacity of HDL and enable it to accept cholesterol via interaction with ATP-binding cassette G1 (ABCG1) receptors expressed by macrophages [135, 136]. Liver X receptor (LXR) regulates the expression of cellular ABCA1 and ABCG1 via heterodimerization with retinoid X receptor (RXR) [137]. Cholesterol transported by HDL is taken up by hepatic scavenger receptor class B type1 (SR-B1) in the liver [138, 139]. Cholesteryl ester transfer protein (CETP) mediate transfer of cholesteryl esters from HDL to apoB-containing lipoproteins (LDL or VLDL) in exchange for triglycerides [140]. ApoA1 has been shown to reduce vascular inflammation in *in vitro* and *in vivo* studies [141, 142]. Recently a mimetic peptide of apoA1 with phospholipids, ETC-642 was shown to decrease adhesion molecule expression in collared rabbit carotid arteries, suppress inflammation via NF-κB signalling pathway [143].

Liver and intestine are the major organs responsible for HDL synthesis, dysfunction of either of these organs under inflammation can lead to decreased profile of plasma HDL [144-146]. During infection and inflammation serum levels of HDL and apoA1 are decreased [10-13]. The proinflammatory cytokine TNF, which plays a major role in the pathogenesis of CD has been shown to decrease HDL cholesterol efflux to apoA1 by attenuating intestinal ABCA1 expression [147-150]. Consistent with this, clinical studies show a significant decrease in HDL-cholesterol in patients with IBD [14]. Furthermore, during inflammatory conditions, HDL is remodelled to an acute phase HDL with reduction in phospholipids and cholesterol esters and an increase in free cholesterol, triglycerides and free fatty acids which increasingly associate with SAA (serum amyloid A) and is proinflammatory [151-153].

1.8 Autophagy - a survival mechanism during cellular stress

Autophagy refers to an evolutionary conserved cellular degradation mechanism that involves engulfment of cytoplasmic contents, delivery to the lysosome where they are degraded. It is upregulated in response to signals such as growth factor deprivation, pathogen infection, starvation, ER stress, and accumulation of unfolded proteins. Autophagy has acquired much attention in recent times for its ability to increase cell survival during periods of stress via catabolism of subcellular proteins, organelles and cytoplasmic components that generate free fatty acids, amino acids and nucleotides. Autophagy has been implicated in immunity, aging, cancer, neurodegenerative disorders and infectious diseases [154-156]. Autophagy contributes to immunity by directly removing pathogens, presenting these to adaptive immune system via MHC-II restricted antigen presentation and by supporting lymphocyte survival as well as homeostasis of T-cells, B-cells and Paneth cells and thereby mediate cytokine production [157-160]. Autophagic substrates include long lived proteins such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Also organelles such as mitochondria, endoplasmic reticulum, excess peroxisomes and mature ribosomes are targeted for lysosomal degradation by autophagosomes [161-166].

35 autophagy related (ATG) genes have been identified. Autophagosomes are characterized by double membrane vesicles which can originate from ER, golgi complex, mitochondria or plasma membrane, the formation of which is mediated by ubiquitination-like conjugation systems [167-170]. Microtubule associated protein light chain 3 (LC3) represent the mammalian ortholog of yeast ATG8. Lipidation of ATG8 (LC3) where ATG8 is conjugated to phosphatidyl-ehtanolamine mediates elongation of the isolation membrane and ATG5-ATG12-ATG16 mediates closure and fusion of autophagosomes with lysosomes to form degradative autolysosomes. Under active autophagy, cytoplamic LC3-I (18 Kda) is converted to a membrane bound phosphatidyl-ethonolamine-conjugated form LC3-II (16 Kda) that accumulates on autophagic vesicles [171-173] . Increase in LC3II is measured to quantify active autophagy. The process of autophagy is dependent on Beclin1 and class III phosphatidylinositol 3-kinase (PI3K) [174-176]. In genome wide association studies, three loci containing autophagy related genes (ATG16L1, IRGM and

LRRK2) was shown to associate with CD, also ATG16L1 and IRGM in UC [177-180]. ATG16L1 deficiency in mouse models leads to Paneth cell abnormalities with higher expression of pro-inflammatory mediators [160]. In addition, a role of autophagy in protecting against toxic products of pathogens such as *Vibrio cholerae* cytolysin, *Bacillus anthracis* lethal toxin and *Helicobacter pylori* vacuolating toxin has been described [181-183].

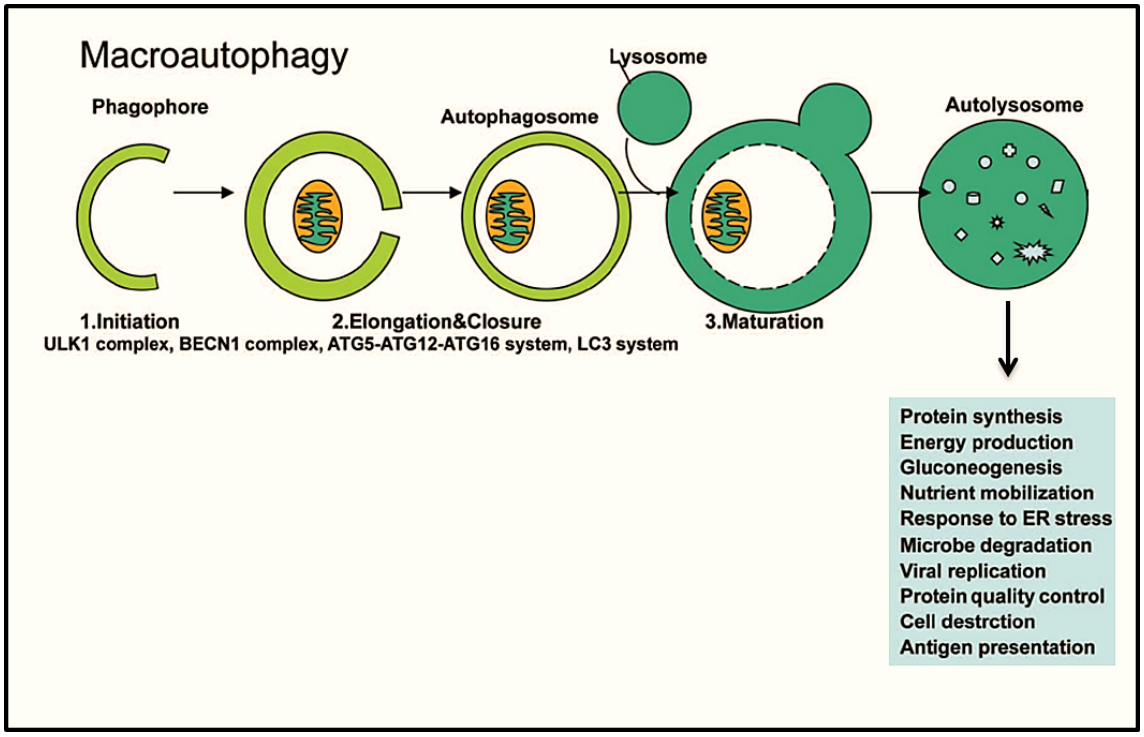


Figure 6: Schematic diagram of macro autophagy

(adapted from [184])

The autophagy system includes Serine/Threonine kinases ULK1 and ULK2 (ATG1) mediating nucleation and activation of autophagy specific class III phosphatidylinositol 3- kinase (PI3K) complex PIK3C3/VPS34-BECLIN1 which recruit two ubiquitin like conjugates, ATG12-ATG5-ATG16L1 complex and LC3 to the outer membrane, which is responsible for elongation of the isolation membrane and enclosure of the autophagosome [173, 185-189]. During this process, the phagophore engulf cytoplasmic constituents including organelles and finally fuse with the lysosome degrading the contents.

2 AIMS OF THE STUDY

The following projects were included in my thesis. The aims of which are listed below:

- In project 1 "**Association of genetic variation in the *NR1H4* gene, encoding the nuclear bile acid receptor FXR, with inflammatory bowel disease**", we aimed to investigate the association between genetic variants of FXR and IBD. Five FXR single nucleotide polymorphisms - two common SNPs and three rare variants were genotyped using Taqman allelic discrimination assays in a well-sized IBD vs. non-IBD cohort.

- Project 2 "**HDL and its major protein ApoA1 suppress intestinal inflammation**" aimed at elucidating the role of HDL/ApoA1 in intestinal inflammation. An *in vitro* model using colon adenocarcinoma cell line T84 was used and an *in vivo* DSS colitis model with ApoA1 knockout and ApoA1 transgenic mice was studied comparative to wild-type mice.

- Project 3 "**Anti-inflammatory effect of HDL and ApoA1 is mediated via activation of autophagic pathways**" was designed to elucidate the molecular pathways by which HDL/ ApoA1 exerts its effects on intestinal cells. Regulation of autophagic pathways by HDL was investigated in T84 cells.

3 METHODS

3.1 Association of genetic variation in the *NR1H4* gene, encoding the nuclear bile acid receptor FXR, with inflammatory bowel disease [103]

Study subjects

The study population was European, and comprised of 591 healthy subjects and 547 IBD patients, from which 203 were diagnosed to suffer from UC and 344 from CD. The IBD subjects were recruited at the centers participating in the Swiss Inflammatory Bowel Disease Cohort Study (SIBDCS) [190]. For the IBD patients, the diagnosis of UC or CD was confirmed by the study investigators based on clinical presentation, endoscopic findings, and histology. Non-IBD controls were recruited from gastroenterological patients undergoing surveillance colonoscopy, and showed no symptoms of IBD. History of colorectal cancer was used as exclusion criteria for both IBD patients and non-IBD controls. All subjects provided their written informed consent to be included in the study. Ethical approvals were obtained from the local medical ethical committees of all study sites involved in the study: 1) The Swiss IBD Cohort Study (SIBDCS) (the participating centers are listed in http://ibdcohort.ch/index.php?id=94&L=2; ethical licence EK-1316). 2) The Bioethical Committee at the Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Warsaw, Poland (ethical licences 25/2006 and 25/2006/2007).

DNA extraction

Genomic DNAs were extracted from either EDTA-blood or intestinal biopsies using the TRIzol reagent (Invitrogen, Basel, Switzerland) or the QIAamp DNA Mini Kit (QIAGEN, Hombrechtikon, Switzerland), respectively, according to the manufacturer's instructions. The genomic DNAs were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and diluted to a final concentration of 10 ng/µl in nuclease free water.

Genotyping of *NR1H4* single nucleotide polymorphisms

Genotyping of the five *NR1H4* SNPs was performed using TaqMan allelic discrimination assays. A 7900HT Fast Real-Time PCR system (Applied Biosystems, Rotkreuz,

Switzerland) was used for cycling. Inventoried TaqMan SNP Genotyping Assays C_28000279_10, C_25598395, C_25598386_10, C_2366616_10, C_2800610_10 for the SNPs *rs3863377*, *rs7138843*, *rs56163822*, *rs35724*, and *rs1086060*, respectively were used for genotyping. Twenty nanograms of each genomic DNA was used per PCR reaction in a volume of 5 µl. The amplification run conditions were: Once 50°C for 2 min, once 95°C for 10 min, 45 times 95°C for 15 sec, and 60°C for 1 min.

Statistical analysis

Statistical analysis for the individual SNP associations was performed using the software package SPSS 18 (SPSS Inc., Chicago, IL). The Chi-square test or Fisher's exact test were used to determine associations between individual SNPs and subject phenotypes. A P-value of <0.05 was considered as significant in non-corrected statistical tests and of <0.01 after correction for multiple testing for the five SNPs (according to Bonferroni). The software package PSPower was used for retrospective power calculations (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). A retrospective power analysis of the applied statistical tests on genotype distributions revealed a power of 0.525 for the SNP *rs3863377* and a power of 0.323 for the SNP *rs56163822*, when considering a Bonferroni-corrected alpha level of 0.01 and the detected ORs as shown in Table 3 for the applied χ^2-tests. Linkage disequilibria (LD) were calculated using D' statistics and the software package Haploview (www.haploview.com). Haplotype predictions and frequency estimations were performed using the software tool FAMHAP (www.famhap.meb.uni-bonn.de). FAMHAP software performs permutation test on associations between estimated haplotypes and the affection state based on Monte Carlo simulations. The expectation maximization (EM) algorithm was used to obtain maximum-likelihood estimates of the haplotype frequencies in a group composed of cases and controls. Individuals with several possible haplotype explanations are assigned with a likelihood weight to each possible haplotype and its calculated frequency estimate. A contingency table is constructed summing up all individuals' weighted haplotype explanations for each haplotype and the chi-square statistics computed. The corresponding P-value is assessed via Monte Carlo simulation, i.e. in each replication of the algorithm a sample composed of a subgroup of case and control samples is randomly drawn and permuted. FAMHAP calculates the global P-value via Monte Carlo simulations, as the cell

counts used in the contingency table are based on haplotype frequency estimates with increased variances, and not on real haplotype counts, which, as a result, does not necessarily follow exactly a chi-square distribution [191, 192]. A value of $P < 0.05$ was considered to be significant. Bonferroni-corrected P-values ($P<0.006$, corrected for eight haplotypes that FAMHAP represented relevant to test for) were defined as the significance level for single haplotype comparisons in the white sections of Tables 4 - 4.2. In addition, haplotypes in best reconstruction (not weighted) were listed for the case and control groups in Tables 4-4.2 (grey sections) and used for association analysis performing Fisher's exact tests or, in case of high cell counts (11 or more), Chi-square tests. Bonferroni-corrected significance levels ($p<0.0026$, corrected for 19 haplotypes) were used for significance testing.

3.2 HDL and its major protein ApoA1 suppress intestinal inflammation

Chemicals

Deoxyadenosine 5′-[α-^{32}P]-triphosphate (6,000 Ci/mmol) was purchased from Perkin Elmer (Schwerzenbach, Switzerland). Unless stated, other chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland),. The luciferase Pathway Profiling System containing the pNF-κB-luc, and the control vector pTAL-luc were obtained from Clontech.

Cell culture

Human colon-derived T84 cells were cultured in DMEM/F-12 (Invitrogen, Basel, Switzerland). The media was supplemented with 10 % fetal bovine serum (FBS; Brunschwig, Basel, Switzerland), and 100 U/ml penicillin / 100 µg/ml streptomycin (Invitrogen). A humidified atmosphere containing 5 % CO_2 at 37 ºC was used for all cell culture.

Isolation of HDL from Plasma

Human HDL (1.063 < d < 1.21 kg/liter) was prepared by sequential ultracentrifugation of fresh normolipidemic plasmas of blood donors [193]. The purity of the lipoprotein preparation was verified by SDS-PAGE to be free of LDL or albumin. HDL was further lyophilized and dissolved in 5M guanidinium-HCl and dialyzed against 1X LDL-buffer (10x LDL buffer composition: 1.12g EDTA, 87.66g NaCl, filled up to 1l with ddH2O, pH-7.4) by centrifugation (20 minutes, 13000 rpm) in microcons (10 Kda cut-off).

RNA isolation, Reverse transcription, and real-time PCR

T84 cells were treated with HDL (50-100 µg/mL) for 18 hours before treating with 25 ng/mL TNF for 3 hours. Total RNA was isolated using TRIzol reagent (Invitrogen). Total RNA from animal tissues was isolated using the RNeasy Mini Kit and the automated sample preparation system Qiacube (Qiagen, Switzerland) as described by manufacturer. One to two µg of RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland), before real-time PCR analysis using the TaqMan assays Hs00174128_m1 (hTNF), Hs00174103_m1 (hIL8), Hs00164932_m1 (ICAM), Hs00222677_m1, human ACTB (beta-actin) endogenous control (Applied Biosystems), Mm00443258_m1 (mTNF), Mm00516023_m1 (mICAM),

4352341E_mACTB, murine (beta-actin) endogenous control for animal experiments, on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Constitutively expressed beta actin was measured as an internal standard for normalization. Comparative threshold cycle method was used to calculate the relative mRNA levels. The cycling conditions for Quantitative real time PCR was 95°C for 20s, 40 cycles of 95°C 1s and 60°C for 20 s with the TaqMan Fast Universal Mastermix. Within an experiment, all tests were performed in triplicates. The mRNA levels obtained in control conditions were set to one and other conditions are shown relative to this as well as to endogenous control beta-actin.

Luciferase reporter assays

To study the effect of HDL on TNF induced NF-κB responsive promoter activity, cells were co-transfected with 400 ng of the luciferase reporter constructs of NF-κB and 200ng of the expression plasmids pTAL-luc at a ratio of 3 μl FuGENE HD per 1 μg DNA. pcDNA3.1(+) vector was added, where appropriate to normalize the amount of DNA transfected. To control for transfection efficiency, 100 ng of the renilla luciferase (pRL-CMV) reporter plasmid (Promega) were co-transfected. Cells were harvested 36 hours after transfection, and luciferase activity determined using the Dual Luciferase Assay System (Promega) and a Luminoskan Ascent microplate luminometer (Thermo Fisher Scientific, Wohlen, Switzerland). Reporter activities obtained for the empty pGL3basic corresponding to respective test condition, as well as for the test construct containing the promoter of interest, in the control conditions, were set to one and fold activities are shown relative to this. All experimental conditions were performed in triplicates and the experiments repeated for at least three times.

Electrophoretic mobility shift assays (EMSAs)

Oligonucleotides with 5-GATC overhangs were used for annealing NF- κB consensus for radioactive labelling by fill in reactions as described [194].

Top strand: 5-GATCAGTTGAGGGGACTTTCCCAGGC-3

Bottom strand: 5-GATCGCCTGGGAAAGTCCCCTCAACT-3

Five micrograms of nuclear protein prepared by using the NE-PER extraction kit (Pierce, Lausanne, Switzerland) was mixed with 50,000 cpm (1.0 ng) of the radioactive probe, and

protein-DNA complexes were allowed to form for 30 minutes at 30°C. In supershift experiments, 1 µg of the anti-NF- κB p65 antibody (C-20; Santa Cruz Biotechnology, SantaCruz, CA) was added to the extracts before binding reactions and incubated at 4°C for 1 hour prior to addition of radioprobes. The EMSA gels were run in 0.5 X Tris-borate-EDTA at 200 V and processed as described for autoradiography [194].

Animals

Control strains designated wild type (WT), C57BL/6J, ApoA1 knockout (KO), B6.129P2-Apoa1tm1Unc/J (-/-) mice and ApoA1 transgenic (Tg), C57BL/6-Tg(APOA1)1Rub/J mice were purchased from Jacksons laboratories (Bar Harbor, Maine 04609 USA).

Induction of acute colitis

Animal experiments were approved by the veterinary authorities of Zurich, Switzerland and were carried out according to Swiss animal welfare laws. Seven- to eight week-old female C57BL/6J-Fue mice (wild type, WT), female ApoA1 knockout mice (ApoA1 KO) and female ApoA1 transgenic (Tg) were used for the experiments and housed in a specified pathogen-free facility in individually ventilated cages. Acute colitis was induced with 2.5% of Dextran sodium sulfate (DSS) (MP Biomedicals, Illkirch, France) in drinking water [195]. The animals were divided into six groups, three DSS groups and three water control groups with six individuals each in such a way that the average weight was similar between the groups. Animals were fed food and water with or without DSS ad libitum.

Determination of colonoscopy score

For colonoscopy scoring we determined mucosal bleeding, abundant fibrin, altered vascular pattern, nontransparent mucosa (granularity), and stool composition. Animals were anesthetized intraperitoneally with a mixture of 90–120 mg/kg body weight ketamine (Vétoquinol, Bern, Switzerland) and 8 mg/kg body weight xylazine (Bayer, Lyssach, Switzerland), and were examined as described previously [196, 197]. The endoscope was introduced with a lubricant (2% lidocaine) through anus in the sedated mouse, the colon was gently inflated with air and photographs of the colon were taken. Recording was performed with the Karl Storz Tele Pack Pal 20043020 (Karl Storz Endoskope, Tuttlingen, Germany).

Determination of histological score and colon length

From the distal third of the colon, 1 cm of colonic tissue was removed and fixed in 4% formalin overnight. Sections of the paraffin-embedded tissue were used for histological analysis, as described previously based on the loss of crypts, loss of goblet cells, infiltration of lymphocytes, thickening of submucosa/mucosa. [195, 198] Histological examination was performed by an independent, blinded investigator.

Myeloperoxidase activity assay

Colon specimens were washed with 1× PBS and homogenized with a tissuelyzer (Qiagen) in 50 mM phosphate buffer (pH 6.0) and 0.5% hexadecyltrimethylammonium bromide (Sigma Aldrich). Three freeze and thaw cycles were performed. Homogenates were then centrifuged for 2 min at maximum speed; 20 µl of the supernatant was mixed with 280 µl of 0.02% dianisidine (in 50 mM phosphate buffer, pH 6.0, and 0.0005% H_2O_2; Sigma Aldrich) and transferred to a 96-well plate in duplicates. After 20 min, absorbance was measured at 460 nm. Protein concentration of the supernatant was determined by bicinchoninic acid protein assay (BCA). Myeloperoxidase (MPO) activity (indicated as arbitrary units) was calculated as mean absorbance (460 nm) per incubation time per protein concentration in grams.

Statistical analysis

The cell-based experiments were repeated for at least three times independently, and representative experiments are shown. All quantitative data are shown as means ± standard deviations of n experiments. Differences between experimental groups were analyzed by one-way ANOVA with Tukey's post hoc test. A p value <0.05 was considered significant. For the correlation of mRNA expression levels in human tissue material, logarithmic values of mRNA levels were analyzed using linear regression. All statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). For animal study, six animals could be analyzed per group. Statistical analysis of weight loss, colon length, histological score, colonoscopy score and MPO was performed by ANOVA and a Post Hoc Multiple Comparison by Tukey.

3.3 Anti-inflammatory effect of HDL and ApoA1 is mediated via activation of autophagic pathway

Western blotting

Cells were grown on 6 well plates for 24 hours, followed by incubation with HDL or ApoA1 for 18 hours prior to treatment with 25 ng/mL TNF for 3 hours. Cells were washed once with 1X PBS and then harvested into Ripa buffer with complete mini (protease inhibitor)

Transfection of cells with siRNAs

Cells were seeded at 400000 cells per well onto 24-well plates, and transfected with ABCA1, SRB1 and LC3 SiGenome SMARTpool (Dharmacon, Lafayette, CO) or siCONTROL non-targeting siRNA #2 (Dharmacon) at a final concentration of 50 nM using the TransIT-TKO reagent (Mirus Bio LLC, LabForce). The transfections were repeated after 24 hours, and the cells were harvested in 1 ml TRIzol (Invitrogen), 24 hours after the second transfection.

Isolation and Culture of Human Colonic Lamina Propria Fibroblasts (CLPF)

Primary human CLPF cultures were derived from surgical specimens of patients with active CD and cultured as described previously [199]. The fibroblasts were cultured in DMEM containing 10% FCS, penicillin (100IU/mL), streptomycin (100 µg/mL), ciprofloxacin (8 µg/mL), gentamycin (50µg/mL), and amphotericin B (1 µg/mL). Genomic DNA was isolated from cultured fibroblasts and genotyped for ATG16L1 variant using predesigned genotyping assays (Applied Biosystems, Foster City, CA) and TaqMan technology. Written informed consent was obtained before specimen collection, and studies were approved by the local Ethics Committee.

Confocal Microscopy

After treatment of cells with HDL for 18 hours and TNF for 3 hours, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes. For LC3 staining, fixed cells were washed with PBS, permeabilized in 100% methanol at -20°C for 10 minutes, washed wit PBS and blocked with 3% BSA for I hour at room temperature. Cells were

subsequently incubated with LC3 antibody (1:200) dilution overnight at 4°C. After 3 TBST washes, cells were incubated with Alexa 488 conjugated secondary rabbit antibody for 1 hour at room temperature and then washed three times in TBST before mounting with antifade DAKO. Cells were observed with a CLSM Leica SP5 laser scanning confocal microscope and analyzed using Leica confocal software (LAS-AF Lite).

Gold labeling of HDL

Colloidal gold was prepared as described [200] using a particle size of 8 nm. For the conjugation of HDL to colloidal gold, 10 ml of a monodisperse gold solution was added to 16 µL lipoprotein solution containing 50 mg lipoprotein protein/ml in phosphate buffer and pH adjusted to 5.5. Excess of lipoprotein was removed by centrifugation at 9,000 *g* for 20 min against glycerine and dialysed in EDTA buffer to remove glycerine.

Incubation of HT29 with gold labeled HDL

Inorder to follow the uptake of HDL into intestinal cell line, colon carcinoma HT29 cells were incubated with gold labeled HDL for 18 hours. The cells were washed in PBS and fixed with 2.5% glutaraldehyde + 0.8% formaldehyde in 50mM NaCL and 0.1M Phosphate buffer for 2 hours at room temperature, post fixed in 1% OsO_4 (50mM Phosphate buffer) at 4°C for one hour, block stained with 1% Uranyl acetate in water for 1 hour after which the tissue was dehydrated in ethanol (70-100%, 30 minutes) and propylene oxide (2 X 20 minutes), polymerized and embedded in epon araldite. Electron microscopy was performed on a Philips CM100 transmission electron microscope.

4 RESULTS

4.1 Association of genetic variation in the *NR1H4* gene, encoding the nuclear bile acid receptor FXR, with inflammatory bowel disease [103]

Study population

The study population was European and consisted of more women (806) than men (332). Detailed demographic data is given in Table 1.

Table 1 Demographic data of the population included in the analysis (adapted from [103])

Characteristics	non-IBD	IBD	CD	UC
Population	591 (51.9%)	547 (48.1%)	344 (30.2%)	203 (17.8%)
Mean age (± SD)	55.7 (± 12.6)	42.6 (± 15.1)	40.9 (± 14.9)	45.4 (± 14.9)
Median age	59	41	39	44
Minimum age	20	16	16	18
Maximum age	81	82	79	82

NR1H4 sequence variability

All five *NR1H4* variants selected for the study are single nucleotide substitutions, previously identified within the *NR1H4* gene (www.ncbi.nlm.nih.gov/snp/). Three of these SNPs can be considered as rare variants: *rs3863377*, *rs56163822*, *rs7138843*, with reported minor allele frequencies (MAF) of 4%, 2.2%, and 0.9%, respectively. The other two variants, *rs10860603* and *rs35724*, are common SNPs, with minor allele frequencies of 20.5% and 40.8% (www.ncbi.nlm.nih.gov/snp/) in European individuals. The obtained allele and genotype frequencies are given in Tables 2 and 3, respectively.

Genetic variation in the *NR1H4* gene and IBD

The *NR1H4* SNP variants *rs3863377* and *rs56163822* were found to be significantly associated with IBD when considering an uncorrected significance level of $p<0.05$. Upon statistical analysis of the allele (Table 2) and genotype (Table 3) frequencies, we observed that the *NR1H4* variant *rs3863377* is significantly less frequent in IBD cases than in non-IBD controls (allele frequencies: P=0.004; wild-type vs. SNP carrier genotype frequencies: P=0.008) even when considering an Bonferroni-corrected significance level of $P<0.01$. Upon subgrouping the IBD patients, the significance of the inverse association of the *rs3863377* SNP remained for the CD patients when considering an uncorrected significance level of $P<0.05$ (allele frequencies: P=0.015; wild-type vs. SNP carrier genotype frequencies: P=0.024), but not for the UC group (allele frequencies: P=0.075; wild-type vs. SNP carrier genotype frequencies: P=0.083). Conversely, the variant *rs56163822* is less prevalent in non-IBD subjects than in IBD patients (allele frequencies: P=0.027; wild-type vs. SNP carrier genotype frequencies: P=0.035); an observation, which is, however, not significant, when considering a corrected significance level of $P<0.01$. Upon subgrouping the patient cohort according to IBD subtypes, the uncorrected association remained only significant for the UC group (allele frequencies: P=0.036; wild-type vs. SNP carrier genotype frequencies: P=0.034). Upon adjustment for age and gender, the uncorrected significance ($P<0.05$) of genotype frequency association with IBD remained for *rs3863377*, but was reduced to $P>0.05$ for *rs56163822*. There were no significant differences in the allele frequency distribution between the subject groups for *NR1H4* variants *rs7138843*, *rs10860603*, and *rs35724*.

Table 2 Allele frequencies of the *NR1H4* variants in the study population

SNP *rs3863377*, (G → A)

Case	MAF (%)	p	OR	CI	Reported MAFs[1]
non-IBD	32 (2.9)				4.0%
IBD	10 (1.1)	0.004**	0.37	0.18-0-75	
CD	6 (1.1)	0.015*	0.36	0.15- 0.86	
UC	4 (1.2)	0.075	0.38	0.14-1.10	

SNP *rs56163822*, (G →T)

Case	MAF (%)	p	OR	CI	Reported MAFs[1]
non-IBD	20 (1.7)				2.2%
IBD	34 (3.2)	0.027*	1.86	1.06-3.25	
CD	20 (2.9)	0.081	1.74	0.93-3.25	
UC	14 (3.5)	0.036*	2.07	1.03-4.13	

SNP *rs7138843*, (A → T)

Case	MAF (%)	p	Reported MAFs[1]
non-IBD	17 (1.7)		0.9%
IBD	21 (2.4)	ns	
CD	11 (2.0)	ns	
UC	10 (3.0)	ns	

SNP *rs10860603*, (G → A)

Case	MAF (%)	p	Reported MAFs[1]
non-IBD	173 (14.9)		20.5 %
IBD	139 (12.9)	ns	
CD	90 (13.4)	ns	
UC	49 (12.3)	ns	

SNP *rs35724*, (G → C)

Case	MAF (%)	p	Reported MAFs[1]
non-IBD	466 (40.0)		40.8%
IBD	471 (43.8)	ns	
CD	299 (44.4)	ns	
UC	172 (43.0)	ns	

MAF, minor allele frequencies; [1]According to the National Center for Biotechnology Information (NCBI) SNP database *, p<0.05; **, p<0.01 , p', p value adjusted to age and gender

OR, odds ratio; CI, confidence interval, WT: wildtype, Het: heterozygous SNP carrier, Hom: homozygous SNP carrier (adapted from [103])

Table 3 Genotype frequencies of the *NR1H4* variants in the study population

SNP *rs3863377* (G→ A)

	WT [GG]	Het [GA]	Hom [AA] (%)	p	p'	OR	CI	χ^2
non-IBD	506 (94.4)	28 (5.2)	2 (0.4)					0.14
IBD	438 (97.8)	10 (2.2)	0	0.008**	0.022*	0.39	0.19-0.80	0.01
CD	271 (97.8)	6 (2.2)	0	0.024*	0.031*	0.37	0.15-0.91	0.01
UC	167 (97.7)	4 (2.3)	0	0.083	0.066	0.40	0.14-1.16	0.01

SNP *rs56163822* (-1G→ T)

	WT [GG]	Het [GT]	Hom [TT] (%)	p	p'	OR	CI	χ^2
non-IBD	560 (96.6)	20 (3.4)	0					0.04
IBD	505 (93.9)	33 (6.1)	1 (0.09)	0.035*	0.451	1.83	1.04-3.23	0.00
CD	319 (94.4)	19 (5.6)	1 (0.09)	0.115	0.846	1.67	0.88-3.17	0.00
UC	186 (93.0)	14 (7.0)	0	0.034*	0.154	2.11	1.04-4.26	0.13

SNP *rs7138843* (A→ T)

	WT [AA]	Het [AT]	Hom [TT] (%)	p	p'	OR	CI	χ^2
non-IBD	495 (96.7)	17 (3.3)	0					0.03
IBD	417 (95.2)	21 (4.8)	0	0.248	0.772	1.47	0.76-2.82	0.06
CD	261 (96.0)	11 (4.0)	0	0.603	0.612	1.23	0.57-2.66	0.04
UC	156 (94.0)	10 (6.0)	0	0.122	0.981	1.87	0.84-4.16	0.10

SNP *rs10860603* (G→ A)

	WT [GG]	Het [GA]	Hom [AA] (%)	p	p'	OR	CI	χ^2
non-IBD	424 (72.8)	143 (24.6)	15 (2.6)					0.49
IBD	407 (75.8)	121(22.5)	9 (1.7)	0.261	0.384	0.857	0.655-1.12	0.00
CD	254 (75.4)	76 (22.5)	7 (2.1)	0.403	0.611	0.877	0.645-1.19	0.22
UC	153 (76.5)	45 (22.5)	2 (1.0)	0.312	0.521	0.824	0.567-1.19	0.43

SNP *rs35724* (C→ G)

	WT [GG]	Het [GC]	Hom [CC] (%)	p	p'	OR	CI	χ^2
non-IBD	217 (37.3)	264 (45.4)	101 (17.3)					1.78
IBD	177 (32.9)	251 (46.7)	110 (20.4)	0.125	0.209	1.213	0.948-1.55	1.47
CD	111 (32.8)	155 (45.9)	72 (21.3)	0.175	0.279	1.216	0.917-1.61	1.68
UC	66 (33.0)	96 (48.0)	38 (19.0)	0.277	0.233	1.207	0.860-1.69	0.09

OR: odds ratio; CI: confidence interval

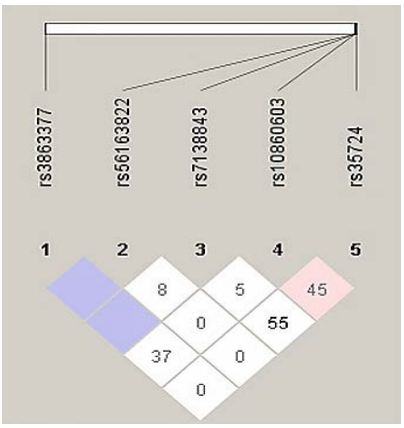
WT: wildtype, Het: heterozygous SNP carrier, Hom: homozygous SNP carrier, χ^2, Hardy-Weinberg equilibrium

χ^2 tests were carried considering hetero- and homozygous SNP carriers as one group in case and control populations

*, p<0.05; **, p<0.01 , p', p value adjusted to age and gender (adapted from [103])

NR1H4 haplotype analysis (adapted from [103])

All individuals, for whom genotype determination could be performed for all five *NR1H4* SNPs under study, were included in the haplotype prediction analyses. Four hundred and eighty-six non-IBD cases, along with 241 CD patients and 148 UC patients were thus haplotyped. Nineteen haplotypes and up to thirty-nine diplotypes were predicted by the software FAMHAP to exist in the studied cohort. *NR1H4* haplotypes were significantly differentially distributed in the IBD and control groups (Table 4, P=0.003) upon global haplotype distribution analysis. This observation held partially true upon stratification according to disease subtype. Here, the haplotype frequencies differ significantly between the UC patients and the non-IBD control group (Table 4.1, P=0.004), but not between the CD patients and the control subjects (Table 4.2, P=0.079). We particularly note that the haplotype 14, *GTTGC*, is predicted to occur significantly (P=0.005) more frequently in the UC group in comparison with the non-IBD cohort. This haplotype harbours the more frequent allele *G* at the first SNP position *rs3863377*, which was shown to be significantly associated with IBD even after Bonferroni correction. It is thus a possible risk haplotype for the development of IBD, although we note that the overall frequency for this haplotype is rather low. Upon best reconstruction analysis the significance of the association of the haplotype 14 *GTTGC* with the UC group was, however, lost (P=0.012) (Table 4.1, grey section). No significant associations were observed for the predicted diplotype patterns and IBD. As shown in the LD plot (Fig 7), there was no significant linkage disequilibrium between any of the five *NR1H4* SNPs studied.



A plot with D' values

Colour scheme:
D' < 1 and LOD < 2, white
D' = 1 and LOD < 2, blue
D' < 1 and LOD ≥ 2, pink

Figure 7: Pairwise linkage disequilibrium calculations between the five *NR1H4* SNPs under study in the non-IBD population

Table 4. Haplotype analysis of IBD patients and non-IBD controls

Order	Haplotype a)b) c)d)e)	Cases (%)	Controls (%)	OR (C.I.)	P	Order	Haplotype a)b) c)d)e)	Cases (%)	Controls (%)	OR (C.I.)	P
1	G G A G G	405.9 (52.2)	510.1 (52.6)	0.98 (0.81-1.19)		1	G G A G G	417 (53.6)	530 (54.6)	0.96 (0.79-1.16)	[a] ns
2	G G A G C	243.9 (31.3)	261 (26.9)	1.24 (1.01-1.53)	0.064	2	G G A G C	235 (30.2)	241 (24.7)	1.31 (1.06-1.62)	[a] 0.012
3	G G A A C	55.9 (7.2)	91.3 (9.4)	0.75 (0.53-1.05)	0.091	3	G G A A C	63 (8.1)	108 (11.1)	0.7 (0.51-0.97)	[a] 0.03
4	G G A A G	27.4 (3.5)	47.5 (4.9)	0.71 (0.44-1.14)		4	G G A A G	18 (2.3)	31(3.2)	0.72 (0.40-1.29)	[a] ns
5	A G A G G	2.7 (0.4)	13.4 (1.4)	0.25 (0.07-0.93)	0.017	5	A G A G G	2 (0.3)	13 (1.3)	0.19 (0.04-0.84)	0.017
6	A G A G C	6.6 (0.8)	12 (1.2)	0.68 (0.26-1.76)		6	A G A G C	8 (1.0)	14 (1.4)	0.71 (0.30-1.7)	ns
7	G G T G C	5 (0.6)	9.1 (0.9)	0.68 (0.23-2.04)		7	G G T G C	4 (0.5)	9 (0.9)	0.55 (0.17-1.8)	ns
8	G T A G G	3.8 (0.5)	7.5 (0.8)	0.63 (0.18-2.17)	NA	8	G T A G G	2 (0.3)	7 (0.7)	0.35 (0.07-1.71)	ns
9	G T A G C	10.4 (1.3)	5.7 (0.6)	2.27 (0.82-6.32)	0.09	9	G T A G C	12 (1.5)	6 (0.6)	2.52 (0.94-6.74)	ns
10	G G T G G	2 (0.3)	3.1 (0.3)	0.79 (0.13-4.70)	NA	10	G G T G G	1 (0.1)	2 (0.2)	0.62 (0.06-6.89)	ns
11	G T A A G	0.8 (0.1)	1.8 (0.2)	NA	NA	11	G T A A G	1 (0.1)	2 (0.2)	0.63 (0.06-6.89)	ns
12	A G A A G	0.2 (0)	2.0 (0.2)	0.13 (0-11.93)	NA	12	A G A A G	0	1 (0.1)	NA	ns
13	G G T A C	2.4 (0.3)	2.3 (0.2)	1.29 (0.21-7.99)	NA	13	G G T A C	4 (0.5)	4 (0.4)	1.25 (0.31-5.0)	ns
14	G T T G C	7.4 (1)	1.0 (0.1)	NA	NA	14	G T T G C	7 (0.9)	1 (0.1)	8.8 (1.08-71.70)	0.026
15	G G T A G	1.1 (0.1)	0.5 (0.1)	NA	NA	15	G G T A G	1 (0.1)	0	NA	ns
16	G T T A G	1.2 (0.2)	1.0 (0.1)	NA	NA	16	G T T A G	3 (0.4)	1 (0.1)	3.75 (0.39-36.15)	ns
17	A G A A C	0.1 (0)	0.6 (0.1)	NA	NA	17	A G A A C	0	0	NA	NA
18	A T A G C	0.4 (0.1)	0 (0)	NA	NA	18	A T A G C	0	0	NA	NA
19	G T T A C	1 (0.1)	0 (0)	NA	NA	19	G T T A C	0	0	NA	NA

389 cases (778 haplotypes) and 485 controls (970 haplotypes) were included into the analysis

Global haplotype distribution: P=0.003

Left table side (white): Predicted haplotypes obtained with Monte Carlo simulations using FAMHAP. The calculation of odds ratios and a global P value for haplotype distribution was performed on these results using FAMHAP. The haplotype order follows a priority ranking within the control group. Nineteen haplotypes were predicted. The haplotype base positions correspond to a) *rs3863377*, b) *rs56163822*, c) *rs7138843*, d) *rs10860603* and e) *rs35724*. As FAMHAP tested all haplotypes with a frequency >0.01, the frequency distribution of eight haplotypes (1-7, 9) was included. Relevant P-values for four haplotypes were displayed by FAMHAP. None of the individual haplotypes are significantly differentially distributed between the IBD and control groups.

OR, odds ratio; NA, not applicable, FAMAHP did not calculate an odds ratio on these results because of a low haplotype frequency value predicted.

Right table side (grey): Most likely occurring haplotype frequencies (haplotype in best reconstruction) predicted by FAMHAP. Based on these predictions significance tests were performed. P-values were calculated using the Fisher's exact test or (if marked with [a]) using the Chi-Square test. A Bonferroni-corrected P-value of 0.0026 (19 tests) was taken as significance level. According to this, none of the haplotypes appeard to be significantly differently distributed between the case and control groups.

OR, odds ratio; C.I., confidence interval; NA, OR not applicable because of at least one cell count with the value of null; ns, not significant and >0.05.

Table 4.1 Haplotype analysis of UC patients and non-IBD controls

Order	Haplotype a)b) c)d)e)	Cases (%)	Controls (%)	OR (C.I.)	P	Order	Haplotype a)b) c)d)e)	Cases (%)	Controls (%)	OR (C.I.)	P
1	G G A G G	146.6 (49.5)	507.5 (52.3)	0.89 (0.69-1.16)		1	G G A G G	153 (51.7)	530 (54.6)	0.89 (0.68-1.15)	[a)] ns
2	G G A G C	98.8 (33.4)	263.9 (27.2)	1.34 (1.01-1.77)	0.022	2	G G A G C	94 (31.8)	239 (24.6)	1.42 (1.07-1.89)	[a)] 0.0149
3	G G A A C	16.9 (5.7)	89.5 (9.2)	0.59 (0.35-1.02)	0.01	3	G G A A C	21 (7.1)	111 (11.4)	0.59 (0.36-0.96)	[a)] 0.038
4	G G A A G	14.2 (4.8)	49 (5.1)	0.95 (0.52-1.73)		4	G G A A G	9 (3)	30 (3.1)	0.98 (0.46-2.09)	[a)] ns
5	A G A G G	1.2 (0.4)	14.1 (1.5)	0.28 (0.04-1.79)	0.11	5	A G A G G	1 (0.3)	13 (1.3)	0.25 (0.03-1.91)	ns
6	A G A G C	1.7 (0.6)	11 (1.1)	0.5 (0.10-2.53)		6	A G A G C	2 (0.7)	14 (1.4)	0.46 (0.10-2.06)	ns
7	G G T G C	1.6 (0.5)	9.4 (1)	0.54 (0.10-2.97)		7	G G T G C	1 (0.3)	11 (1.1)	0.3 (0.04-2.3)	ns
8	G T A G G	2.2 (0.7)	7.8 (0.8)	0.92 (0.21-4.13)	NA	8	G T A G G	1 (0.3)	7 (0.7)	0.47 (0.06-3.81)	ns
9	G T A G C	2.6 (0.9)	5.4 (0.6)	1.58 (0.36-6.99)	NA	9	G T A G C	3 (1)	6 (0.6)	1.65 (0.41-6.62)	ns
10	G G T G G	0.3 (0.1)	3 (0.3)	0.37 (0.01-13.09)	NA	10	G G T G G	0	2 (0.2)	NA	ns
11	G T A A G	0.8 (0.3)	1.8 (0.2)	NA	NA	11	G T A A G	1 (0.3)	2 (0.2)	1.64 (0.15-18.17)	ns
12	A G A A G	0.2 (0.1)	2 (0.2)	0.33 (0-30.58)	NA	12	A G A A G	0	1 (0.1)	NA	ns
13	G G T A C	1.1 (0.4)	2 (0.2)	1.83 (0.18-18.47)	NA	13	G G T A C	2 (0.7)	1 (0.1)	6.59 (0.60-73.0)	ns
14	G T T G C	4.4 (1.5)	1 (0.1)	NA	0.005*	14	G T T G C	4 (1.4)	1 (0.1)	13.27 (1.48-119.3)	0.012
15	G G T A G	1.3 (0.4)	0.7 (0.1)	NA	NA	15	G G T A G	1 (0.3)	1 (0.1)	3.29 (0.20-52.71)	ns
16	G T T A G	1.2 (0.4)	1 (0.1)	NA	NA	16	G T T A G	2 (0.7)	1 (0.1)	6.59 (0.60-73.0)	ns
17	A G A A C	0.2 (0.1)	0.9 (0.1)	NA	NA	17	A G A A C	0	0	NA	NA
18	A T A G C	0.7 (0.2)	0	NA	NA	18	A T A G C	1 (0.3)	0	NA	ns
19	G T T A C	0.2 (0.1)	0	NA	NA	19	G T T A C	0	0	NA	NA

148 cases (296 haplotypes) and controls 485 (970 haplotypes) were included into the analysis

Global haplotype distribution: P=0.004

Left table side (white): Predicted haplotype frequencies obtained with Monte Carlo simulations using FAMHAP: The calculation of odds ratios and a global p value for haplotype distribution was performed on these results using FAMHAP. The haplotype order follows a priority ranking within the control group. Nineteen haplotypes were predicted. The haplotype base positions correspond to a) *rs3863377*, b) *rs56163822*, c) *rs7138843*, d) *rs10860603* and e) *rs35724*. As FAMHAP tested all haplotypes with a frequency >0.01, the frequency distribution of eight haplotypes (1-7, 14) was included. Relevant P-values for four haplotypes were displayed by FAMHAP. One single haplotype (14) was significantly differentially distributed between the UC and control groups. OR, odds ratio; NA, not applicable, FAMAHP did not calculate an odds ratio on these results because of a low haplotype frequency value predicted.

Right table side (grey): Most likely occurring haplotype frequencies (haplotypes in best recontruction) predicted by FAMHAP. Based on these predictions significance tests were performed. P-values were calculated using the Fisher's exact test or (if marked with [a)]) the Chi-Square-test. A Bonferroni-corrected P-value of 0.0026 (19 tests) was taken as significance level. According to this, none of the haplotypes appeared to be significantly different distributed between the UC and control groups. OR, odds ratio; C.I., confidence interval; NA, OR not applicable because of at least one cell count with the value of null; ns, not significant and p > 0.05.

Table 4.2 Haplotype analysis of CD patients and non-IBD controls

Order	Haplotype a)b) c)d)e)	Cases (%)	Controls (%)	OR (C.I.)	P	Order	Haplotype a)b) c)d)e)	Cases (%)	Controls (%)	OR (C.I.)	P
1	G G A G G	258.6 (53.6)	511.2 (52.7)	1.04 (0.83-1.29)		1	G G A G G	265 (55.5)	530 (55.3)	1.01(0.81-1.26)	[a)] ns
2	G G A G C	146.8 (30.5)	259 (26.7)	1.20 (0.95-1.53)		2	G G A G C	140 (29.4)	239 (24.9)	1.25 (0.98-1.60)	[a)] ns
3	G G A A C	39.3 (8.2)	93.9 (9.7)	0.83 (0.56-1.22)		3	G G A A C	44 (9.2)	110 (11.5)	0.79 (0.54-1.14)	[a)] ns
4	G G A A G	13 (2.7)	45.9 (4.7)	0.56 (0.3-1.04)	0.062	4	G G A A G	9 (1.9)	31 (3.2)	0.58 (0.27-1.22)	ns
5	A G A G G	2.1(0.4)	14.4 (1.5)	0.29 (0.07-1.24)	0.053	5	A G A G G	1 (0.2)	13 (1.4)	0.15 (0.02-1.17)	0.044
6	A G A G C	3.9 (0.8)	11.6 (1.2)	0.68 (0.22-2.15)		6	A G A G C	5 (1)	14 (1.5)	0.72 (0.26-2.0)	ns
7	G G T G C	3.8 (0.8)	9.4 (1)	0.81 (0.24-2.68)		7	G G T G C	5	11	0.91 (0.32-2.65)	ns
8	G T A G G	2.1 (0.4)	7.8 (0.8)	0.53 (0.11-2.49)	NA	8	G T A G G	1 (0.2)	7 (0.7)	0.29 (0.04-2.33)	ns
9	G T A G C	6.1 (1.3)	5.7 (0.6)	2.19 (0.7-6.91)	0.122	9	G T A G C	7 (1.5)	6 (0.6)	2.37 (0.79-7.09)	ns
10	G G T G G	1.5 (0.3)	3.1 (0.3)	0.98 (0.14-6.86)	NA	10	G G T G G	1 (0.2)	2 (0.2)	1.01 (0.09-11.13)	ns
11	G T A A G	0	1.6 (0.2)	NA	NA	11	G T A A G	0	2 (0.2)	NA	ns
12	A G A A G	0	2.1 (0.2)	NA	NA	12	A G A A G	0	1 (0.1)	NA	ns
13	G G T A C	0.9(0.2)	2.5 (0.3)	0.73 (0.07-7.9)	NA	13	G G T A C	0	2 (0.2)	NA	ns
14	G T T G C	3.1(0.6)	0.9 (0.1)	NA	NA	14	G T T G C	3 (0.6)	1 (0.1)	6.07 (0.63-58.53)	ns
15	G G T A G	NA	NA	NA	NA	15	G G T A G	0	0	NA	NA
16	G T T A G	0.7(0.1)	1.0 (0.1)	NA	NA	16	G T T A G	1 (0.2)	1 (0.1)	2.02 (0.13-32.3)	ns
17	A G A A C	NA	NA	NA	NA	17	A G A A C	0	0	NA	NA
18	A T A G C	NA	NA	NA	NA	18	A T A G C	0	0	NA	NA
19	G T T A C	NA	NA	NA	NA	19	G T T A C	0	0	NA	NA

241 cases (477 haplotypes) and 485 controls (959 haplotypes) were included into the analysis

Global haplotype distribution: P=0.079

Left table side (white): Predicted haplotype frequencies obtained with Monte Carlo simulations using FAMHAP: The calculation of odds ratios and a global p value for haplotype distribution was performed on these results using FAMHAP. The haplotype order follows a priority ranking within the control group. Nineteen haplotypes were predicted. The haplotype base positions correspond to a) *rs3863377*, b) *rs56163822*, c) *rs7138843*, d) *rs10860603* and e) *rs35724*. As FAMHAP tested all haplotypes with a frequency >0.01, the frequency distribution of eight haplotypes (1-7, 9) was included. Relevant P-values for three haplotypes were displayed by FAMHAP. None of the individual haplotypes are significantly differentially distributed between the CD and control groups. OR, odds ratio; NA, not applicable, FAMAHP did not calculate an odds ratio on these results because of a low haplotype frequency value predicted.

Right table side (grey): Most likely occurring haplotype frequencies (haplotypes in best reconstruction) predicted by FAMHAP. Based on these predictions significance tests were performed P-values were calculated using the Fisher's exact test or (if marked with [a)]) the Chi-Square-test. A Bonferroni-corrected P-value of 0.0026 (19 tests) was taken as significance level. According to this, none of the haplotypes appeared to be significantly different distributed between the CD and control groups.

OR, odds ratio; C.I., confidence interval; NA, OR not applicable because of at least one cell count with the value of null; ns, not significant and p > 0.05.

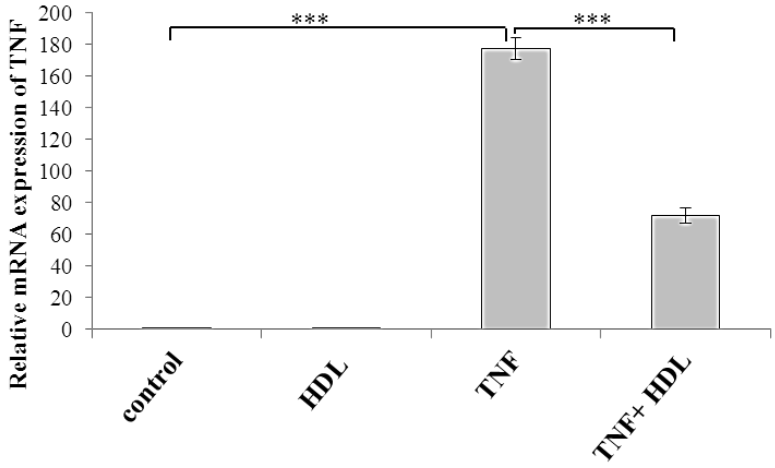
4.2 HDL and its major protein ApoA1 suppress intestinal inflammation

HDL has anti-inflammatory effects *in vitro*

We have used human colon carcinoma cell line T84 which exhibit phenotype similar to mature enterocytes including differentiation into absorptive cells and polarization for *in vitro* experiments.

HDL suppresses pro inflammatory mediators TNF, IL-8 and ICAM in T84 cells.

In order to study potential anti-inflammatory effects of HDL *in vitro*, we treated T84 cells with HDL for 18 hours and subsequently stimulated the cells with 25 ng/mL TNF for 3hrs. As read-out, mRNA expression of pro-inflammatory mediators TNF and IL-8 as well as ICAM was quantified in the presence and absence of HDL. TNF incubation induced mRNA expression of TNF, IL-8, and ICAM by factors 178, 127 and 830, respectively (all $P<0.001$). Pre-treatment of these cells with HDL significantly decreased TNF-induced mRNA expression of TNF, IL-8 and ICAM by 59%, 31% and 50%, (all $P<0.001$) (Fig 8.).



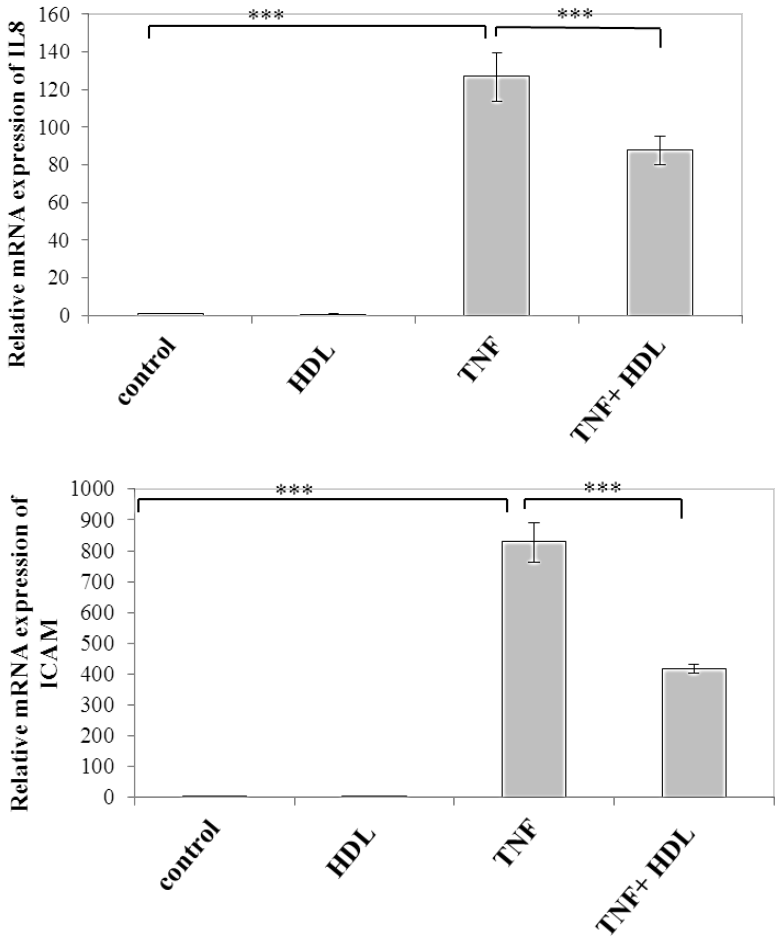


Figure 8 : HDL suppressed mRNA expression of TNF, IL-8 and ICAM-1

Cells were treated with HDL (200 µg/mL) for 18hrs after which 25 ng/mL TNF was added for 3 hours. mRNA expression of TNF, IL8 and ICAM was quantified by RT-PCR and normalized to actin. Each bar represent mean ± SD, n=3, *** P<0.001

HDL suppressed TNF mediated induction of TNF mRNA expression in a concentration dependent manner by 20% in the presence of 50 µg/mL HDL and to 60% in the presence of 200 µg/mL HDL (Fig 9, $P<0.001$).

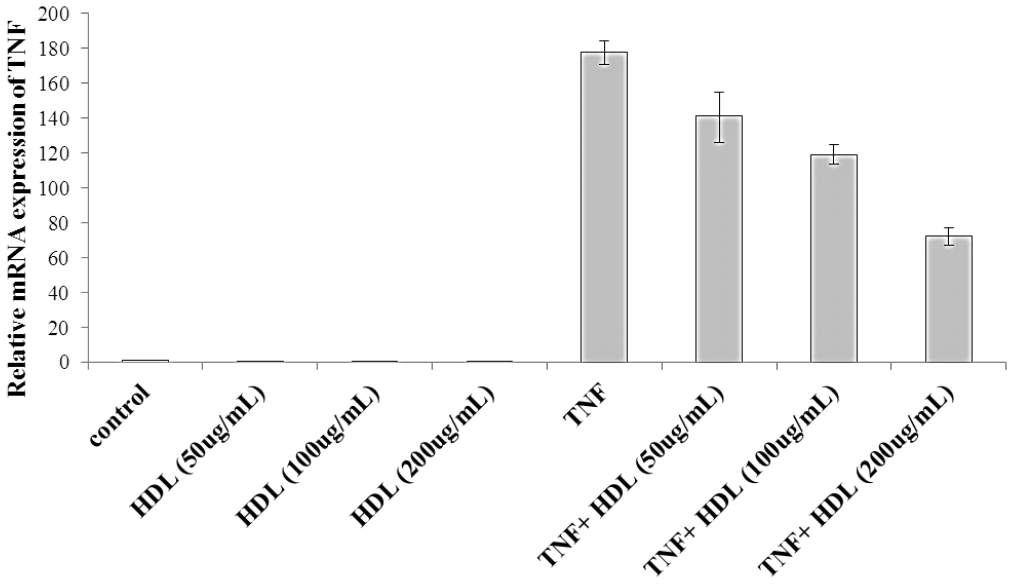


Figure 9: HDL suppressed inflammation in a concentration dependent manner

T84 cells were treated with HDL for 18 h after which 25 ng/mL TNF was added for 3 hours. mRNA expression of TNF was quantified by RT-PCR and normalized to actin. Each bar represent mean ± SD, n=3, *** $P<0.001$

HDL decreases phosphorylation of IκB kinase (IKK)

We further investigated the mechanism by which HDL suppress proinflammatory cytokine secretion. NF-κB is a key transcription factor that regulates genes such as TNF, IL-8 or ICAM, involved in immune and inflammatory responses [201]. In non stimulated cells, NF-κB is held inactive in the cytoplasm bound to the inhibitor protein IκB. The translocation of NF-κB to the nucleus is preceded by phosphorylation of IκB kinase complex (IKK). Upon stimulation with NF-κB inducers such as TNF, IκB is rapidly phosphorylated on its serine residues (S32 and S36) by IKK, ubiquitinated and degraded by 26S proteasome [202, 203]. In order to investigate the role of HDL in the NF-κB pathway, we extracted whole cell protein from T84 cells treated with HDL for 18 h in the presence and absence of TNF (25 ng/mL). HDL decreased phosphorylation of IKK, in T84 cells (Fig 10) slightly but not significantly. TNF preincubation increased phosphorylation

of IKK by 46%. The TNF induced IKK posphorylation was reduced , when the cells were pretreated with HDL by 70%.

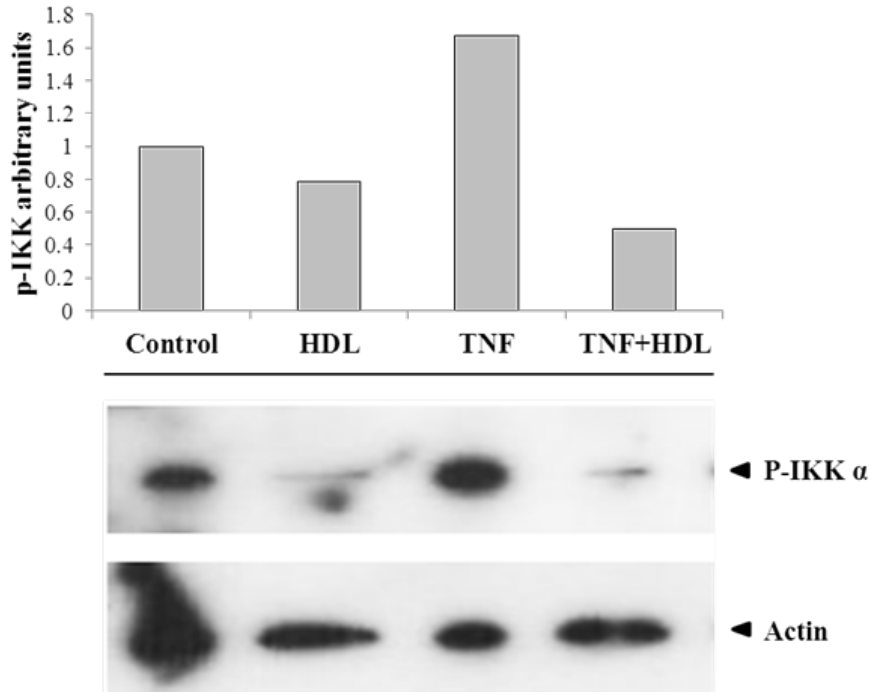


Figure 10: HDL decreased phosphorylation of IKK (IkB kinase)

Whole cell protein extracts from T84 cells were immunoblotted for phosphorylated form of IKK. P-IKK arbitrary units represent ratio of p-IKK to actin and then normalized to the untreated control

HDL suppresses TNF induced NF-κB responsive promoter activity in T84 cells

As HDL prevents TNF induced expression of NF-kB dependent genes and TNF-induced activation of IKK, we next investigated whether HDL also prevents TNF induced NF-κB responsive promoter activity. T84 cells were transfected with luciferase constructs expressing an NF-κB responsive element along with corresponding controls (empty vector-pTAL-luc). 24 hours after transfection, the cells were incubated with HDL (100 µg/mL) for 18 hours, after which 25 ng/mL TNF was added for 6 hours. The cells were harvested and analysed by a dual luciferase reporter assay. As shown in Fig 11, HDL itself inhibited NF-κB luciferase activity by 26% ($P<0.05$). TNF pre-incubation almost tripled luciferase activity in our assay. Preincubation with HDL significantly decreased the TNF induced NF-κB responsive promoter activity by 50% ($P<0.001$).

Very similarly ApoA1 also decreased the effect of TNF stimulation by 50% (P<0.001) indicating that the protein component of the lipoprotein is responsible for mediating the anti-inflammatory effect.

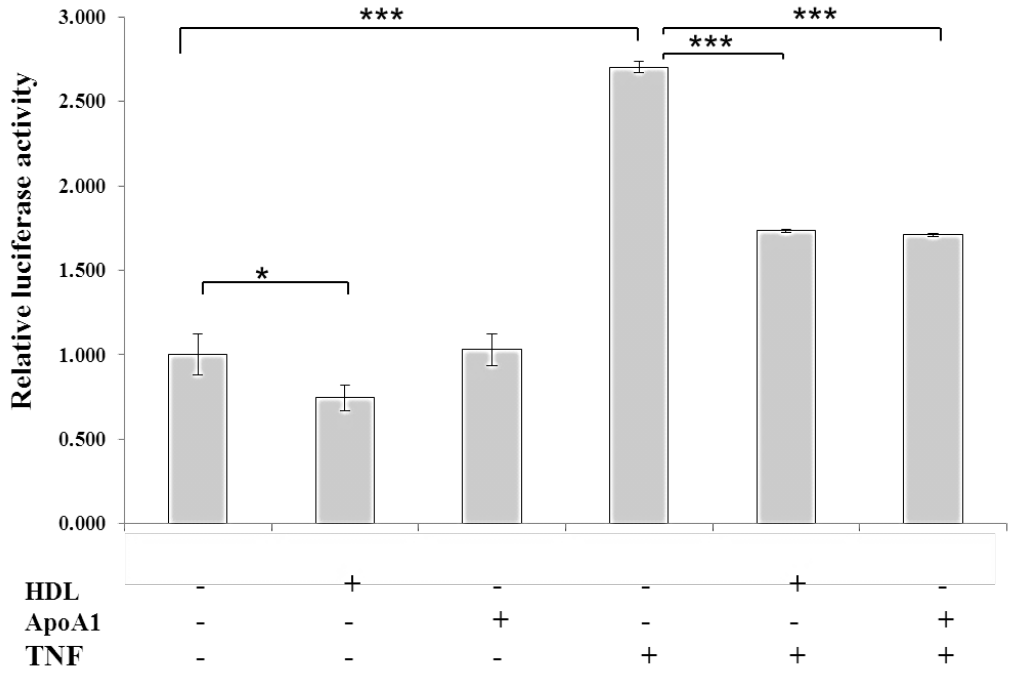


Figure 11: HDL suppresses TNF induced NF-κB responsive promoter activity

T84 cells were transiently transfected with plasmid containing NF-κB responsive promoter region cloned upstream of a luciferase reporter gene. TNF significantly increased NF-κB responsive promoter activity, preincubation of cells with HDL/ApoA1 significantly suppressed TNF induced NF-κB responsive promoter activity. n=3; *, P<0.05, ***, P<0.001.

We further investigated whether pretreatment of T84 cells with HDL affects the nuclear translocation of NF-κB in stimulated or unstimulated cells. As assayed by EMSA, using a radiolabeled NF-κB binding motif consensus, under control conditions as well as pre-treatment with only HDL or ApoA1, no nuclear translocation was visible. As expected TNF caused a strong nuclear translocation of NF-κB (Fig 12, lane 3). Pretreatment of T84 cells with HDL or ApoA1 decreased TNF induced NF-κB DNA binding activity clearly (Fib 12 lanes 4 and 6). A supershift was observed upon incubation of nuclear extracts with the P65 antibody.

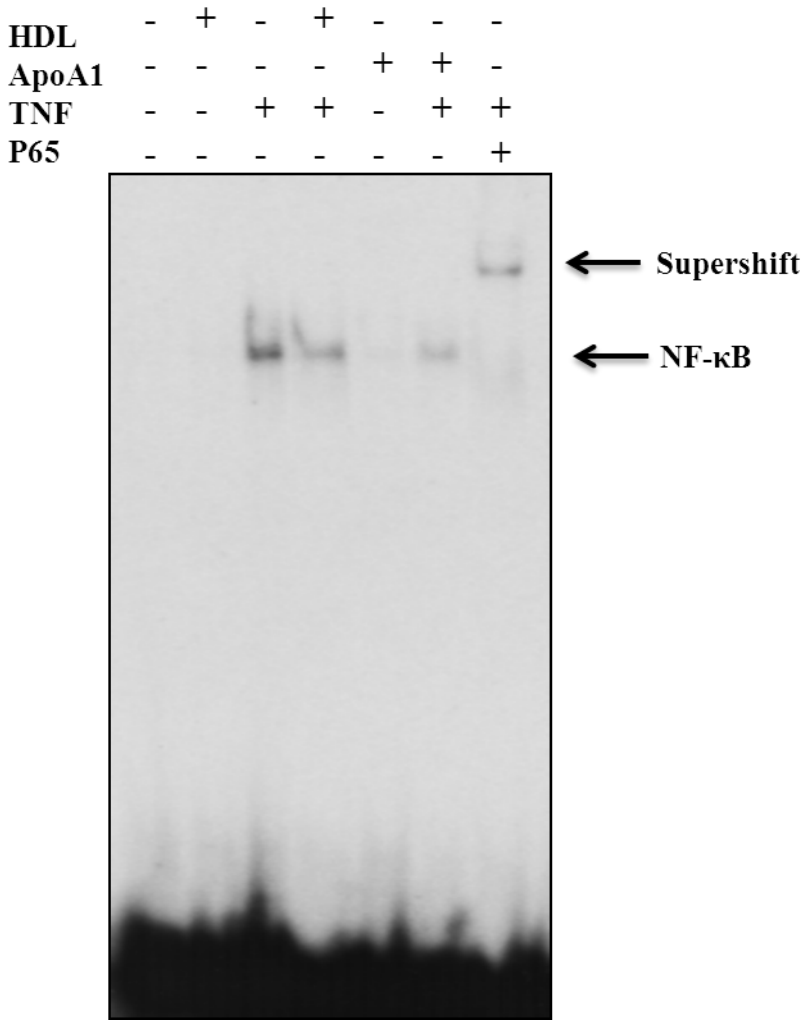


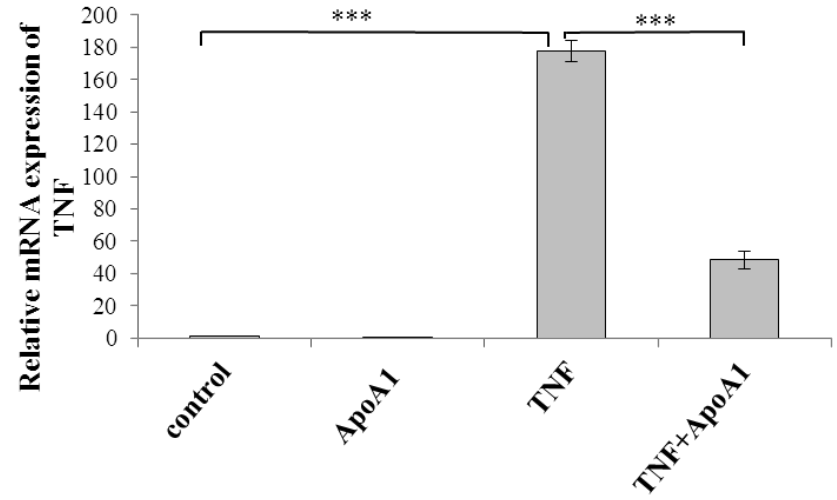
Figure 12: HDL/ApoA1 decreased TNF induced NF-κB DNA binding activity

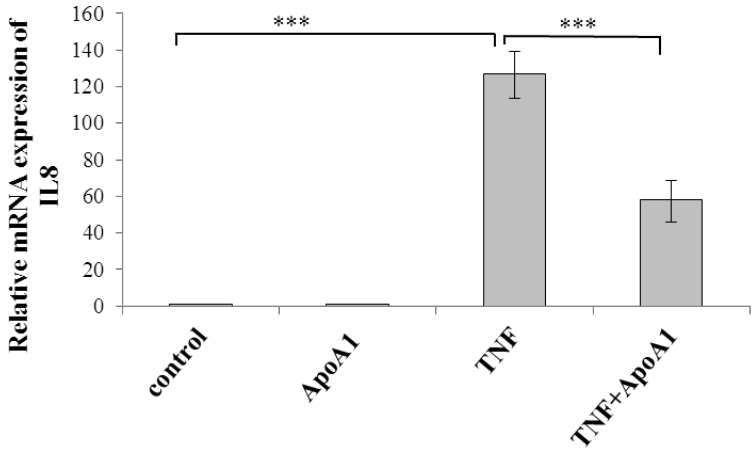
Nuclear extracts from T84 cells were subjected to EMSA using a ^{32}P labelled NF-κB consensus. The arrow head indicates binding reaction of TNF to NF-κB consensus. HDL/ApoA1 decreases TNF binding to NF-κB. A supershift was observed upon incubation of nuclear extracts with P65 antibody; n=3.

The anti-inflammatory effect of HDL is mediated by its main protein ApoA1

ApoA1 is the major protein constituent of plasma HDL. We next tested if pretreatment of T84 cells with ApoA1 could suppress TNF induced NF-κB responsive promoter activity and mRNA expression of target genes. Pretreatment of T84 cells with 100 µg/mL ApoA1 led to a 37% decrease in TNF induced NF-κB responsive promoter activity ($P<0.001$) (Fig 11). mRNA expression of TNF, IL-8 and ICAM was also significantly decreased by 73%,

54% and 51% respectively (P<0.001), when T84 cells were incubated with ApoA1 (prior to TNF addition (Fig 13). The suppression of TNF by ApoA1 was concentration dependent (Fig 14) ranging from 35% in the presence of 50 µg/mL ApoA1 and to 73% in the presence of 200 µg/mL ApoA1 (P<0.001).





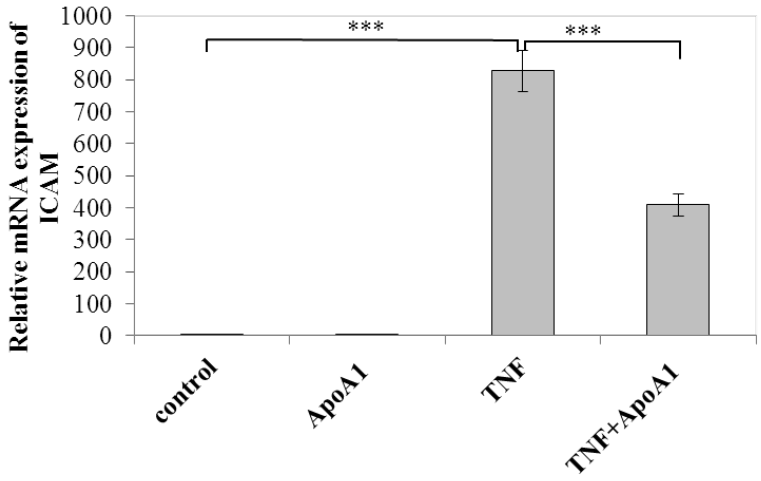


Figure 13: ApoA1 suppresses mRNA expression of TNF, IL-8 and ICAM

Cells were treated with ApoA1 (100 µg) for 18hrs after which 25 ng/mL TNF was added for 3 hours. mRNA expression of TNF, IL8 and ICAM was quantified by RT-PCR and normalized to actin. Each bar represent mean ± SD, n=3, *** P<0.001.

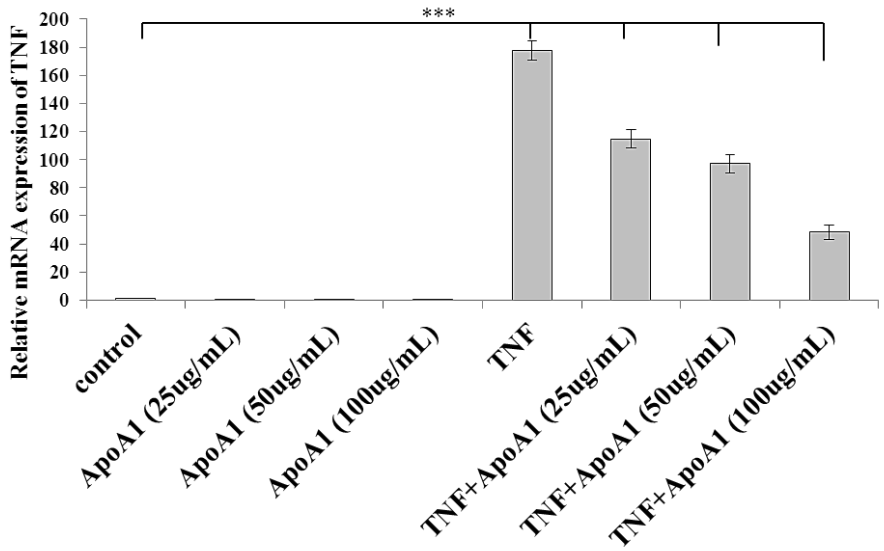


Figure 14: ApoA1 suppresses inflammation in a concentration dependent manner

Cells were treated with ApoA1 (100 µg) for 18hrs after which 25 ng/mL TNF was added for 3 hours. mRNA expression of TNF was quantified by RT-PCR and normalized to actin. Each bar represent mean ± SD, n=3, *** P<0.001.

Weight loss was monitored during the 7 day course of acute colitis. Upon treatment with DSS and in comparison with the water-drunk control animals, the body weight decreased significantly in WT mice and ApoA1 KO mice ($P<0.001$) but not in ApoA1 Tg mice (Fig 15). Colon length was significantly shortened in all animals with DSS treatment compared to their respective water controls. However the shortening was most pronounced in WT mice ($P<0.001$) and ApoA1 KO mice ($P<0.001$) and least pronounced in ApoA1 Tg mice ($P<0.05$).

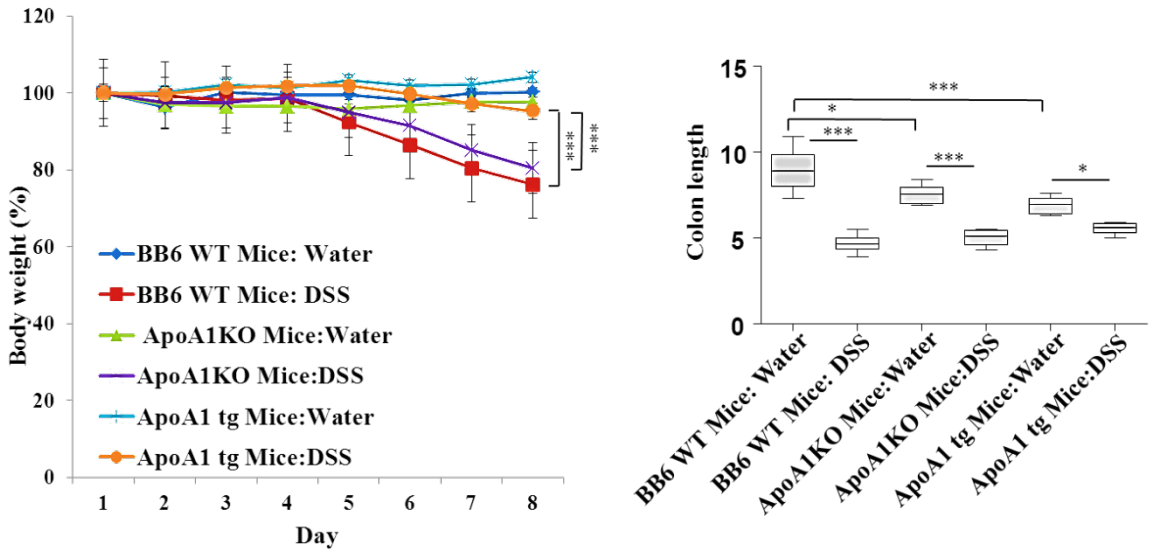


Figure 15: Assessment of clinical colitis parameter: weight loss and colon shortening upon DSS induced colitis

The weight loss was monitored over 8 days, calculated as relative change of body weight in % to day one. ApoA1 KO and WT mice lost more weight than ApoA1 Tg upon DSS. The colon length was significantly decreased in mice recieving DSS within the groups. Each bar represent mean ± SEM, n=6, * $P<0.05$, *** $P<0.001$.

ApoA1 reduces colonic inflammation *in vivo*

To investigate whether ApoAI can protect colitis associated intestinal tissue damage *in vivo*, an acute dextrane sodium sulfate (DSS) colitis model was applied to wild type mice (WT), ApoA1 knock-out mice (ApoAI KO) and mice transgenic for human ApoAI (ApoAI-Tg). Mice received either pure water or water supplemented with 2.5% DSS for a period of 7 days. Mucosal damage was assessed by miniendoscopy and quantified by using the MEICS score (Fig 16).

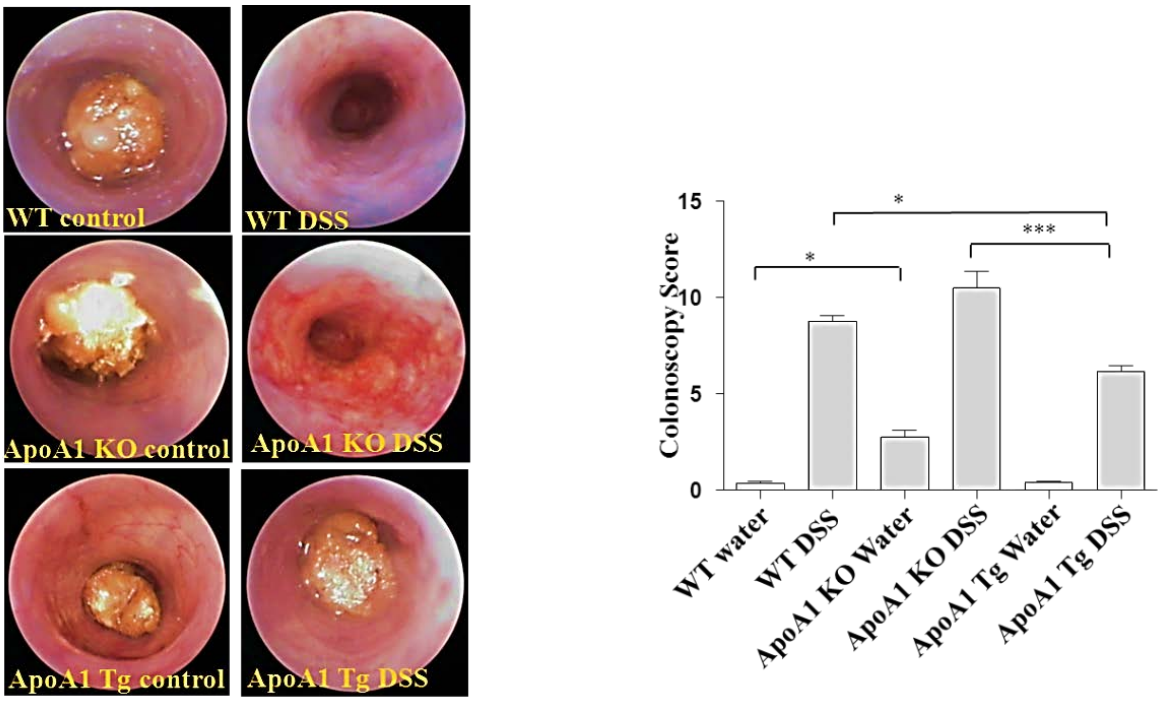


Figure 16: Miniendoscopy of distal colon

Colonoscopy was performed on day 8 prior to sacrificing mice. Representative images of colonoscopy of water and DSS treated mice are shown. Murine endoscopic index of colitis severity (MEICS) was scored based on the images and plotted as box plot. Severe disruption of epithelium was observed in ApoA1 KO mice recieving DSS where as ApoA1 Tg mice displayed an intact surface with still formed stool compared to WT upon DSS colitis. Each bar represent mean ± SEM, n=6, * $P<0.05$, *** $P<0.001$

Interestingly the endoscopic score indicated that the mucosa was significantly damaged in ApoA1 KO mice receiving water only ($P<0.01$) as compared to WT and ApoA1 Tg mice receiving pure water. Mucosa of WT and ApoA1 Tg mice receiving only water displayed a smooth, well vascularized mucosa, a solid stool was visible where as ApoA1 KO mice had low levels of diarrhea and thickening of the mucosal walls. Upon DSS treatment severe mucosal damage occurred in all animals ($P<0.001$) compared to the water controls. Notably ApoA1 Tg mice demonstrated significantly less damage than WT and ApoA1 KO mice upon DSS treatment ($P<0.05$). Upon DSS treatment, ApoA1 KO mice displayed a mucosa with cobblestone-like appearance and increased bleeding as compared to ApoA1 Tg mice.

To assess the severity of colitis, we also analysed distal colon tissue specimens histologically. As shown in Fig 17, a total loss of crypt structure with increased infiltration of lymphocytes into the submucosa and thickening of the bowel wall were observed in the DSS groups as compared to the respective water groups (P<0.001). Upon DSS treatment, the total histological score was significantly increased for ApoA1 KO mice compared with WT mice (P<0.05) and ApoA1 Tg mice (P<0.01). ApoA1 KO mice displayed significantly more infiltration of inflammatory cells compared to WT mice (P<0.001) and ApoA1 Tg mice (P<0.05).

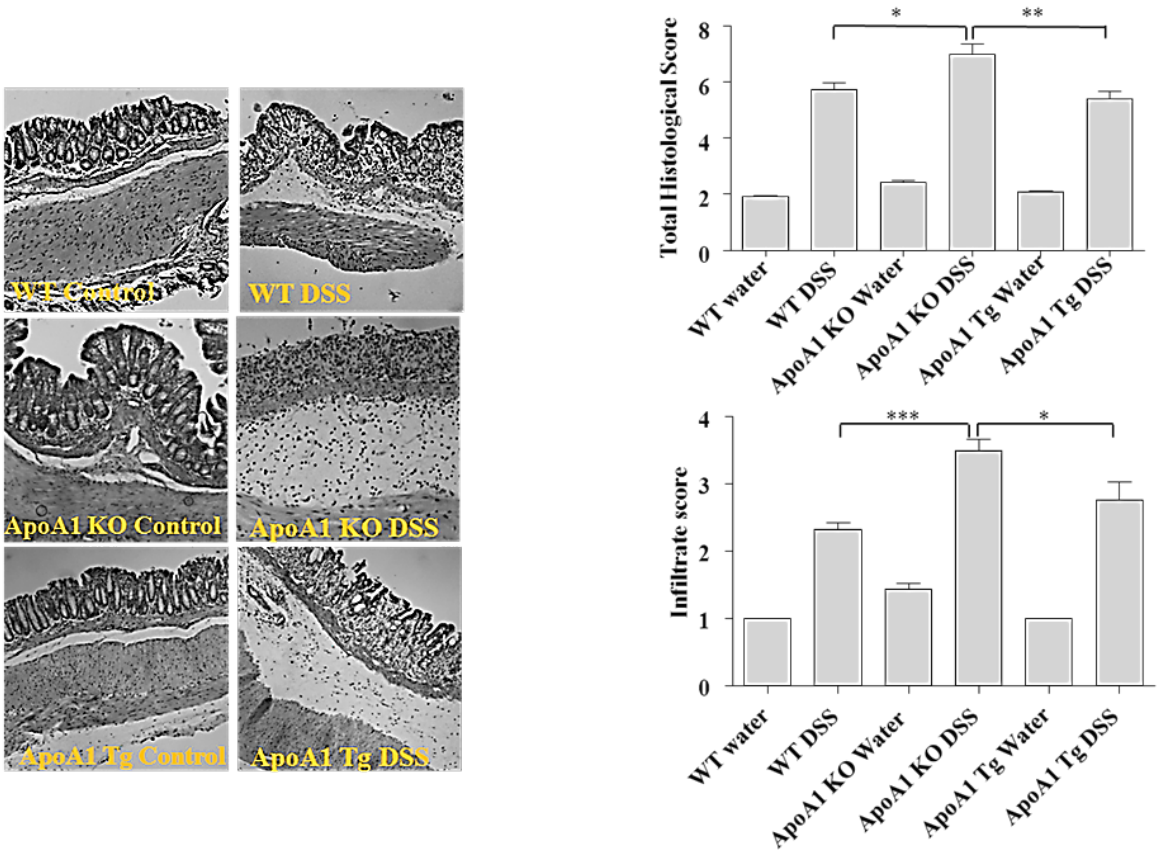


Figure 17: Hematoxylin and eosin (HE) staining of terminal colon

Histological sections were scored based on morphology of epithelia, loss of goblet and crypt cells and infiltration of inflammatory cells. HE section of ApoA1 KO recieving DSS displayed severe barrier breakdown with extensive infiltration reaching the lamina muscularis mucosae. The total histological score calculated as the sum of epithelium and infiltrate score is plotted. Each bar represent mean ± SEM, n=6, * P<0.05, ** P<0.01, *** P<0.001.

Migration of leukocytes to the site of injury is a major event in inflammation. Spectrometric determination of myeloperoxidase (MPO) activity is a quantitative assessment of neutrophil infiltration. The relative MPO activity in tissue homogenates was significantly higher in each DSS group than in its corresponding water group (P<0.001) (Fig 18). However, tissue homogenates of DSS-treated ApoA1 Tg mice showed significantly lower MPO activity than those of DSS-treated ApoA1 KO mice and WT mice (P<0.001).

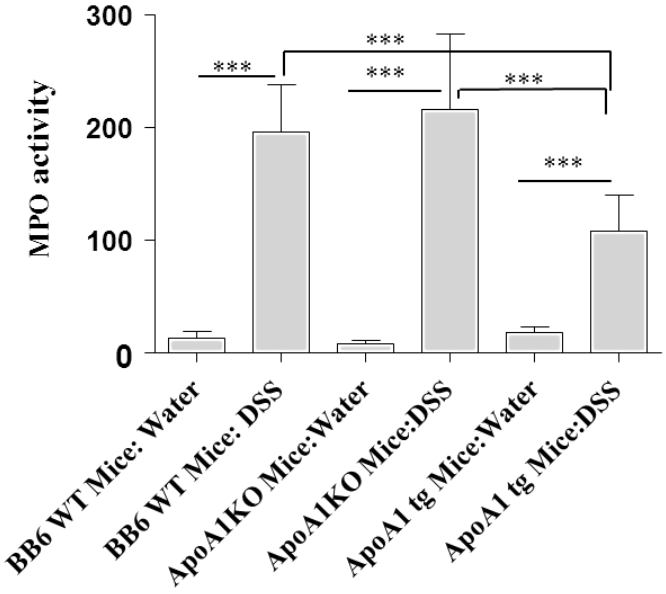


Figure 18: Myeloperoxidase activity in mucosal homogenates of ApoA1 KO, Tg mice comparative to WT mice

Relative MPO activity of each DSS group to its corresponding water groups was significantly increased in WT and ApoA1 KO mice. ApoA1 Tg displayed less MPO activity comparative to WT and ApoA1 KO, however did not reach significance. Each bar represent mean ± SEM, n=6, *** P<0.001.

Increased expression of TNF and ICAM in ApoA1 KO mice

The mRNA expression of the proinflammatory cytokine TNF and the adhesion molecule ICAM was measured in whole colon tissues (Fig 19). Already in the absence of DSS, the expression of TNF and ICAM was increased in ApoA1 KO mice compared to WT mice (P<0.001 for TNF, ns for ICAM). Upon DSS feeding TNF and ICAM mRNA expression

increased significantly in all animals (P<0.001) but most tremendously in ApoA1 KO mice. As the result TNF and ICAM expression in ApoA1 KO mice was 18 and 34 times, respectively, higher than in WT mice and 10 and 168 times, respectively, higher than in ApoA1 Tg mice (both P<0.001).

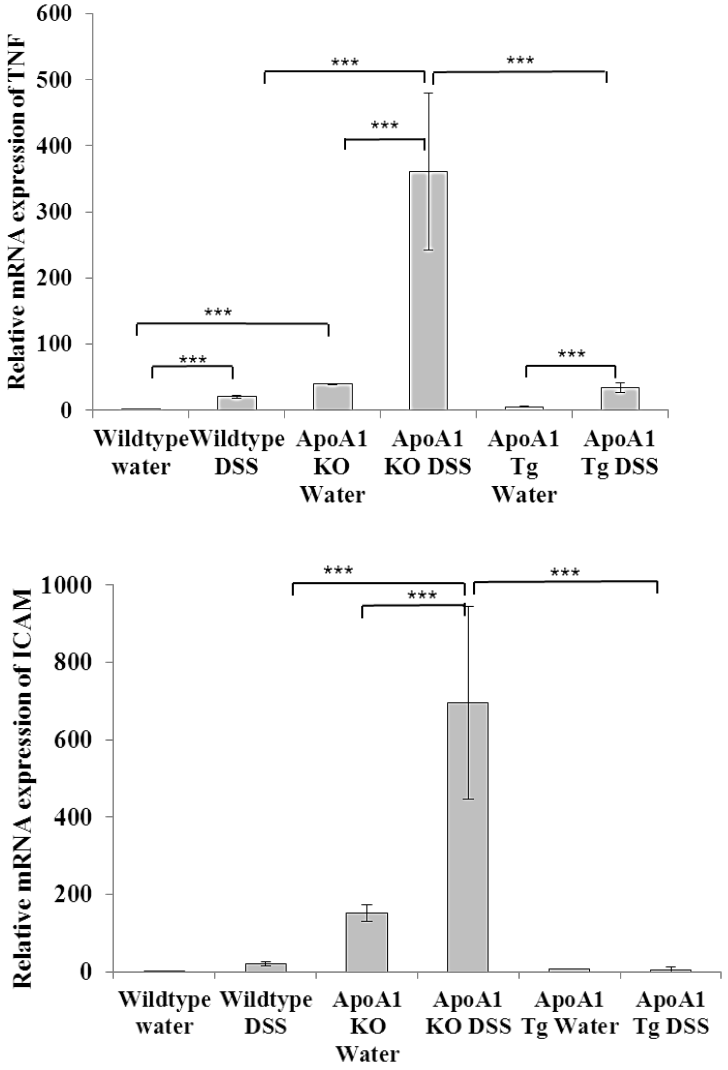


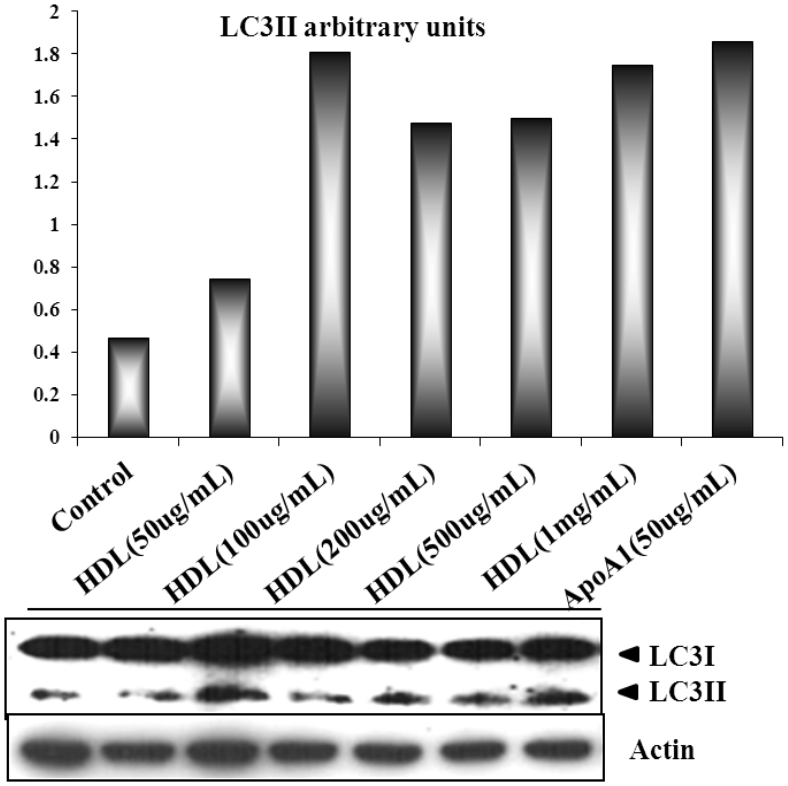
Figure 19: mRNA expression of TNF and ICAM in colon tissue samples of ApoA1 KO , Tg and WT mice with and without DSS administration

ApoA1 KO mice recieving DSS displayed significant increase in mRNA expression of TNF and ICAM where as ApoA1 Tg mice displayed lower expression levels of TNF and ICAM comparative to WT and ApoA1 KO upon DSS induced colitis. The mRNA expression was calculated relative to WT mice recieving water. Each bar represent mean ± SEM, n=6, *** P<0.001.

4.3 Anti-inflammatory effect of HDL and ApoA1 is mediated via activation of autophagic pathway

HDL induces autophagy in T84 cells by inhibiting phosphorylation of mTOR

The phospholipid conjugation of cytosolic protein LC3I to membrane bound LC3-II is a reliable marker for autophagic activation. Autophagy induction was confirmed by western blotting and immunofluorescense analysis with anti-LC3 antibody. HDL induced concentration dependent accumulation of LC3-II (Fig 20). Consistent with this, HDL decreased phosphorylation of mTOR, a negative regulator of the autophagic pathway (Fig 21).



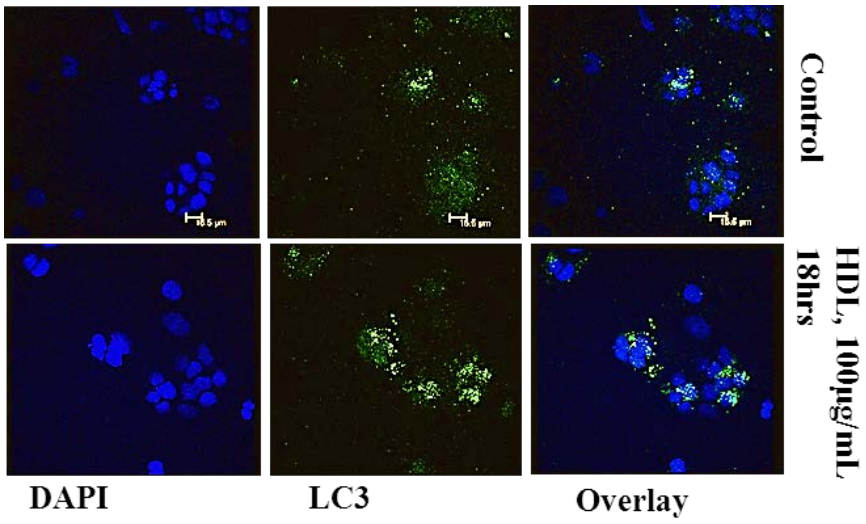


Figure 20: HDL and ApoA1 induces LC3 in T84 cells

T84 cells were incubated with HDL and ApoA1 for 18 hours. Whole cell proteins were extracted in RIPA buffer for western blot analysis. For immunofluorescence, the cells were fixed in 4% formaldehyde and stained for LC3; n=3.

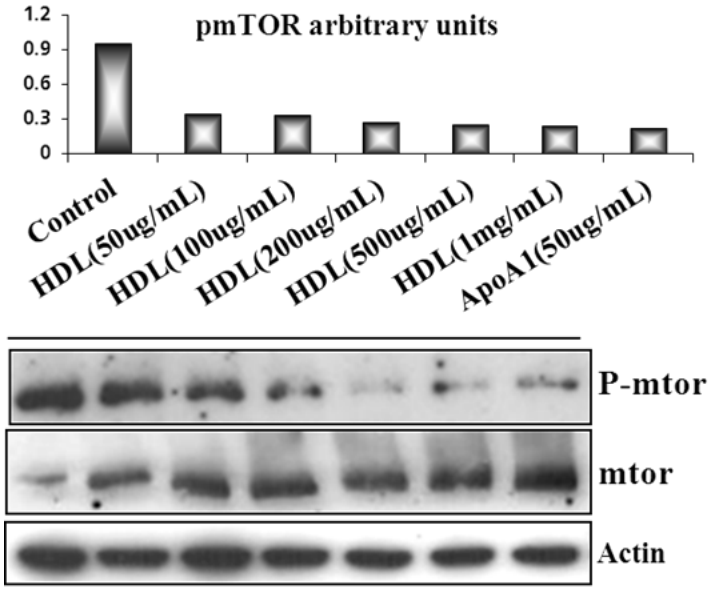


Figure 21: HDL and ApoA1 decreases phosphorylation of mTOR; n=2

Inhibition of autophagy reverses the anti-inflammatory effect of HDL

To confirm that anti-inflammatory effect of HDL is mediated via autophagy, we pre-treated T84 cells with autophagic inhibitors 3-methyl adenine (10mM) and Wortmannin (25nM) for 6 hours prior to HDL addition. The ability of HDL to suppress TNF induced NF-κB responsive promoter activity (Fig 22) as well as mRNA expression of TNF was decreased when autophagy was inhibited (Fig 23). In addition, SiRNA knock down of LC3 decreased the anti-inflammatory effect of HDL, assessed by the ability of HDL to suppress TNF induced mRNA expression of TNF (Fig 24).

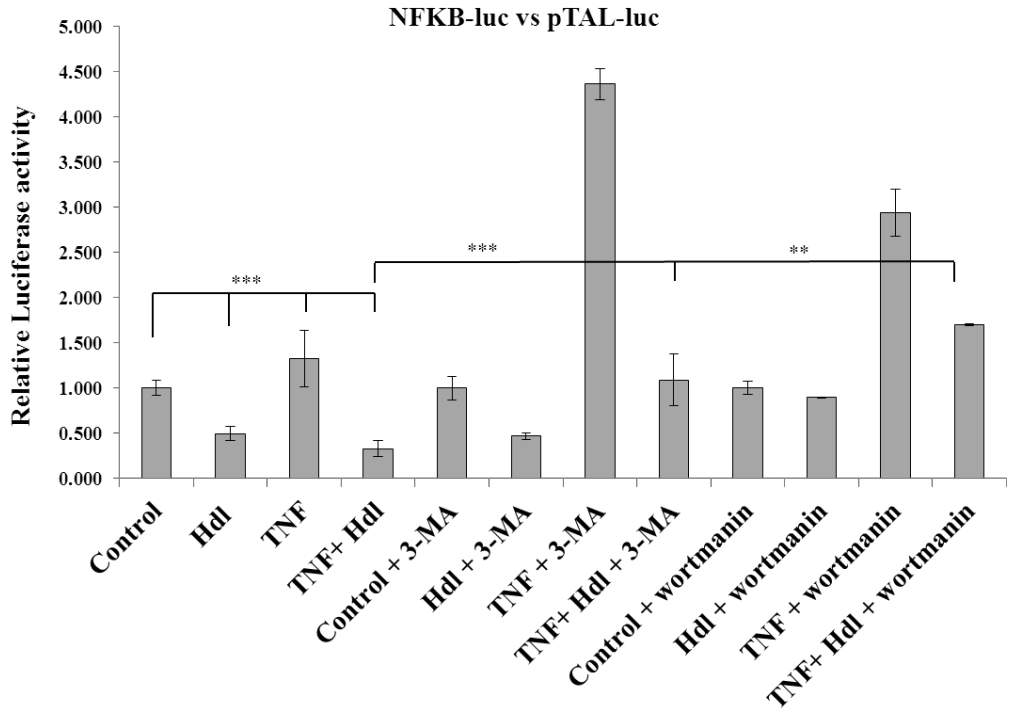


Figure 22: Ability of HDL to suppress TNF induced NF-κB responsive promoter activity is decreased in the presence of PI-3 kinase inhibitor Wortmannin and autophagy specific class III PI-3 kinase inhibitor 3-methyl adenine (3-MA); n=3.

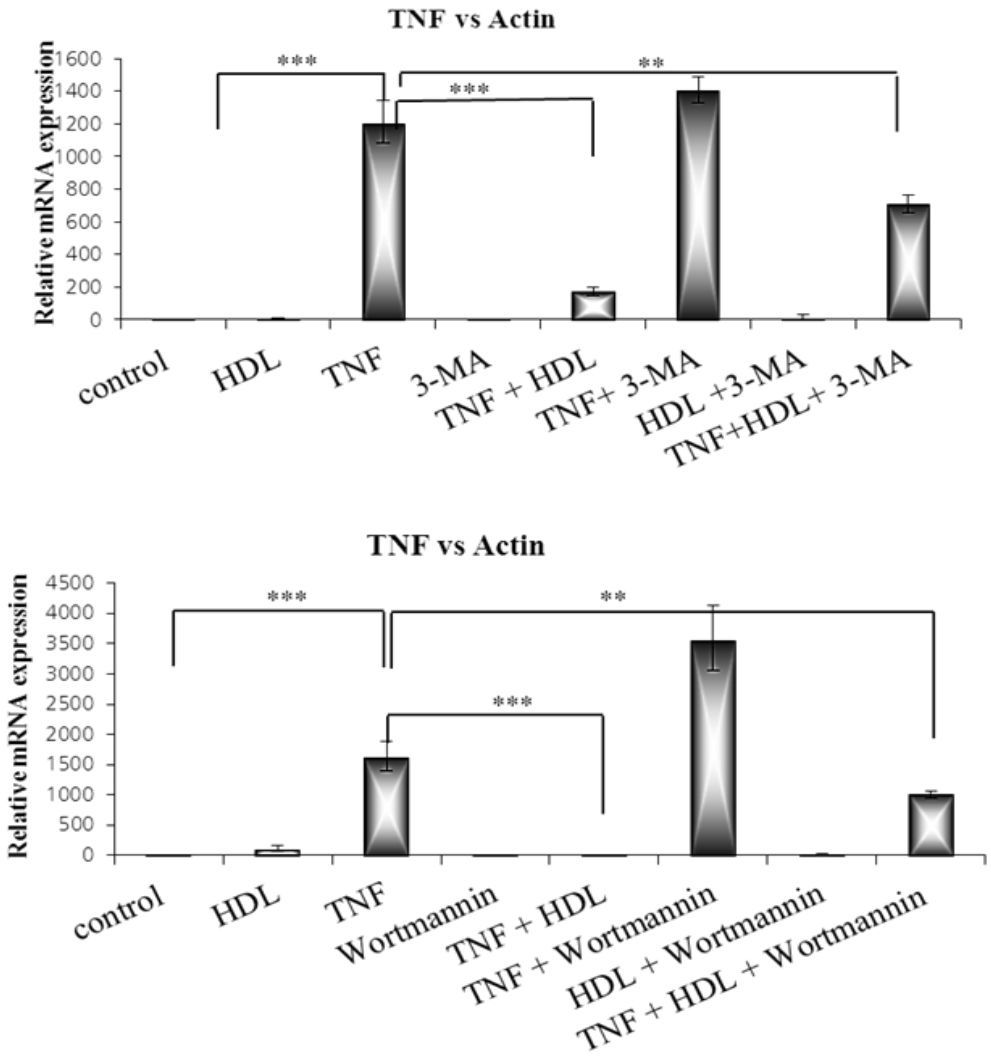


Figure 23: Ability of HDL to suppress TNF induced mRNA expression of TNF is decreased in the presence of autophagy inhibitors

T84 cells were incubated with 10 mM 3-methyladenine or 25 nM Wortmannin for 6 hours to inhibit PI-3 kinase, after which HDL was added for 18 hours prior to treatment with 25 ng/mL TNF for 3 hours. mRNA expression of TNF was quantified relative to actin; n=3.

SiRNA knockdown of LC3 reverses the anti-inflammatory effect of HDL

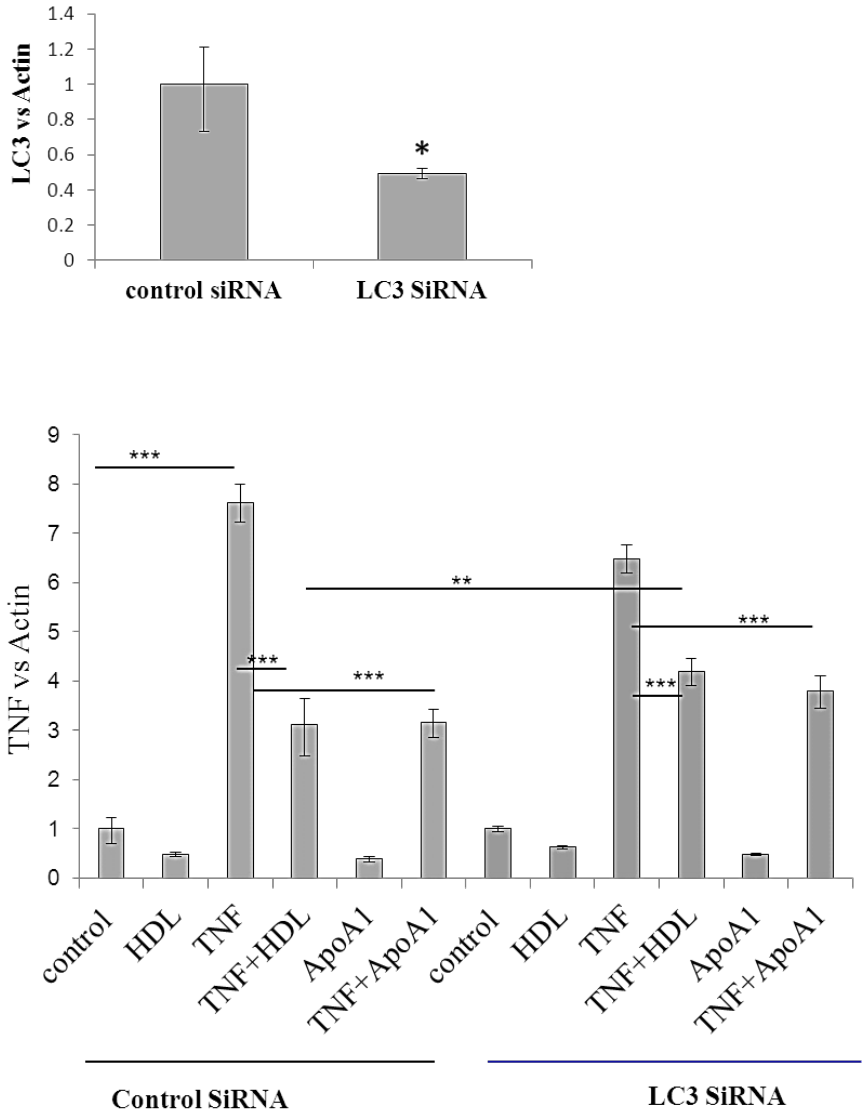


Figure 24: SiRNA knockdown of autophagy gene LC3 decreases the anti-inflammatory effect of HDL

T84 cells were transfected with 50 nM control SiRNA or LC3 SiRNA. 48 hours after transfection, cells were incubated with HDL (100 µg/mL) and ApoA1(50 µg/mL) for 18hrs, after which 25 ng/mL TNF was added to the cells. mRNA expression of LC3 and TNF was quantified relative to actin; n=3.

Anti-inflammatory effect of HDL is reduced in human fibroblast with ATG16L1 mutation

Human fibroblasts harboring heterozygous (A/G) (CU 137) and homozygous (G/G) (CU 138) mutation of ATG16L1 was compared to control fibroblast with both active alleles of ATG16L1 (A/A) (CU 403) to study the significance of autophagy gene ATG16L1 in the anti-inflammatory effect mediated by HDL. As shown in Fig 25 , the mRNA expression of TNF was significantly higher in fibroblast with ATG16L1 mutations upon incubation with TNF, the ability of HDL to suppress TNF induced mRNA expression of TNF was significantly reduced in homozygous and heterozygous variants of ATG16L1 comparative to control. In mutant fibroblast CU138 which has both alleles mutated, anti-inflammatory effect of HDL was significantly decreased comparative to CU137 which has one functional allele.

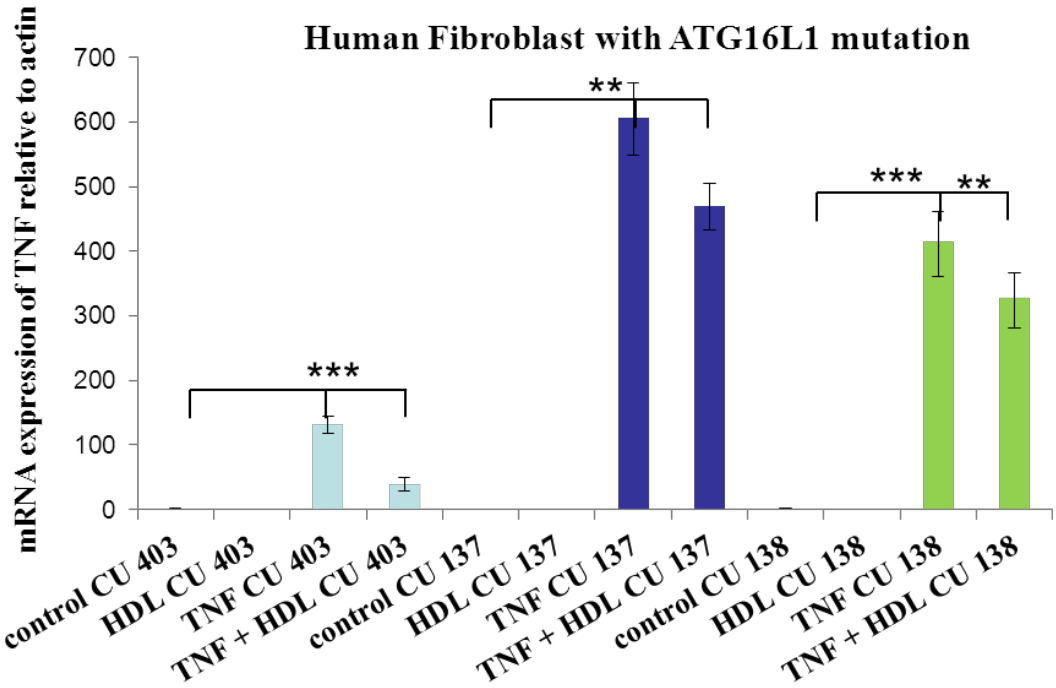


Figure 25: mRNA expression of TNF in human fibroblast with ATG16L1 mutation

Fibroblasts were treated with HDL for 18 hrs after which 25 ng/mL TNF was added to the cells. mRNA expression of TNF was quantified relative to actin; n=3.

Pretreatment of T84 cells with Bafilomycin A1 reversed the anti-inflammatory effect mediated by HDL

Bafilomycin is a selective inhibitor of vacuolar ATP-ase, it increases the pH of acidic vesicles and thereby prevent the fusion of autophagosomes with lysosomes, inhibiting the late stages of autophagy. We next tested if pretreatment of T84 cells with 100 nM Bafilomycin A1 had any effect in the anti-inflammatory effect mediated by HDL. As shown in Fig 26, preincubation of T84 cells with Bafilomycin A1 prior to HDL addition significantly decreased the ability of HDL to suppress TNF induced expression of TNF.

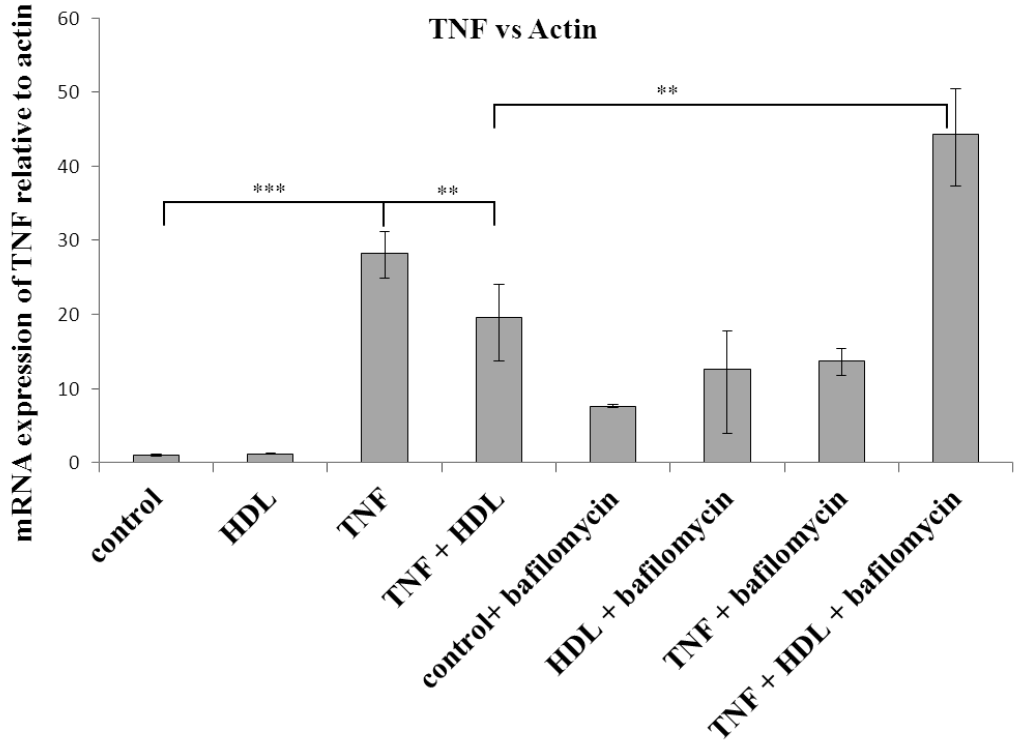


Figure 26: Anti-inflammatory effect of HDL is decreased in the presence of autophagosome fusion inhibitor bafilomycin A1

T84 cells were incubated with 100 nM bafilomycin A1 for 6 hours to inhibit autophagy, after which HDL was added for 18 hours prior to treatment with 25 ng/mL TNF for 3 hours. mRNA expression of TNF was quantified relative to actin; n=3.

Uptake of gold labeled HDL into HT29 cells

When colon carcinoma HT29 cells were incubated with gold labeled HDL for 18 hours, HDL was endocytosed in vesicles resembling autophagosomes with typical characteristics of engulfed cytoplasmic material and organelles (Fig 27).

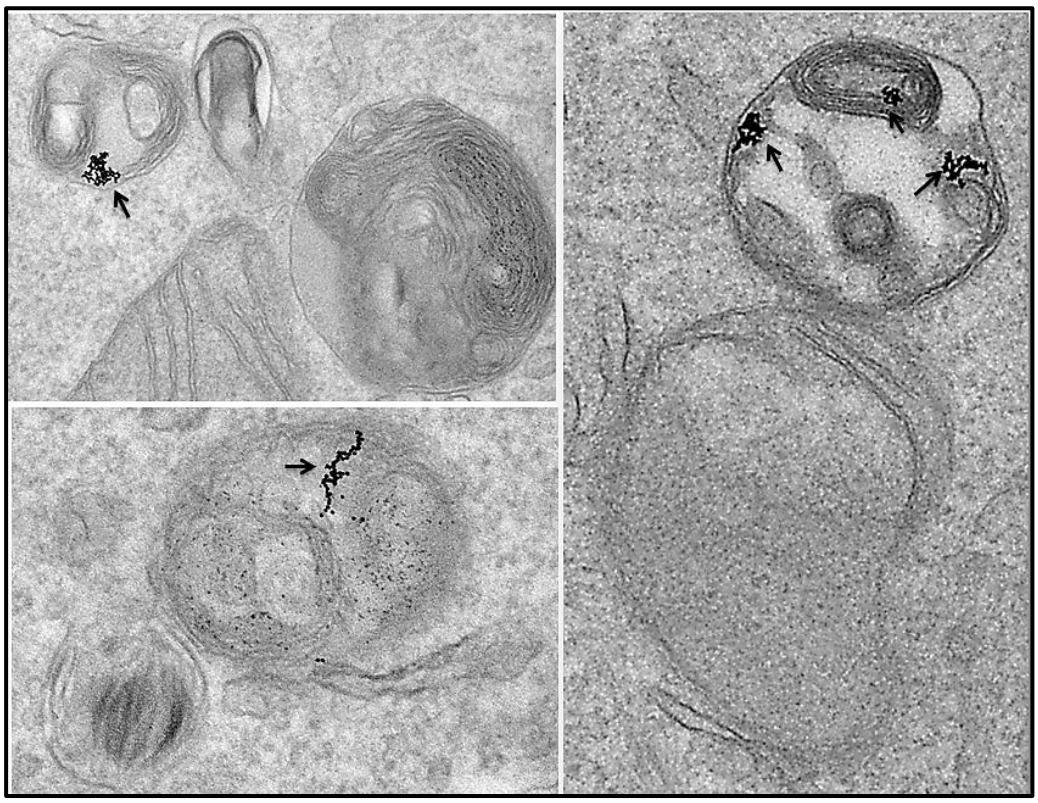
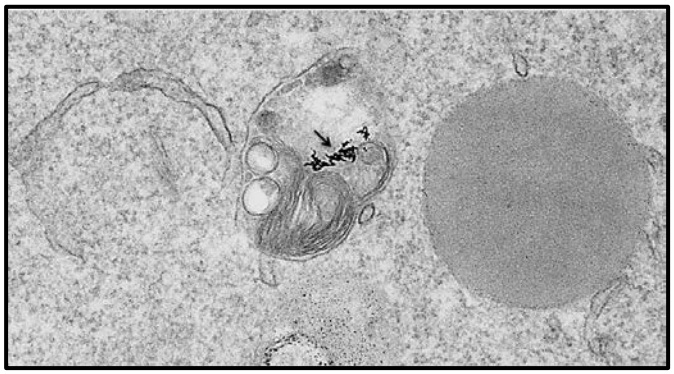


Figure 27: Uptake of gold labeled HDL into autophagosome

HT29 cells were incuabated with 8nm gold labeled HDL for 18 hours, after which the cells were fixed and embedded in epon araldite for electron microscopy.

The arrow represent gold labeled HDL. The organelle in close proximity to autophagosome could possibly be lysosomes.



Lipid droplet in vicinity of an autophagosome displaying a possibility of cholesterol exchange between autophagosome and lipid droplet.

Intracellular transport and anitiinflammatory effect of HDL is mediated by tubulin

Tubulin has been shown to mediate intracellular transport of HDL. Inorder to study the role of microtubules in the anti-inflammatory effect mediated by HDL, T84 cells were incubated with 5 µM colchicine which binds tubulin and block its polymerisation prior to HDL addition. As shown in Fig 28, colchicine significantly (n=3) decreased the ability of HDL to suppress TNF induced expression of TNF. With regard to the proposed autophagy mechanism, inhibition of tubulin might hinder efficient uptake of HDL by autophagosomes, there are also studies showing that autophagosomes might also exploit tubulin for their intracellular transport and fusion with lysosomes.

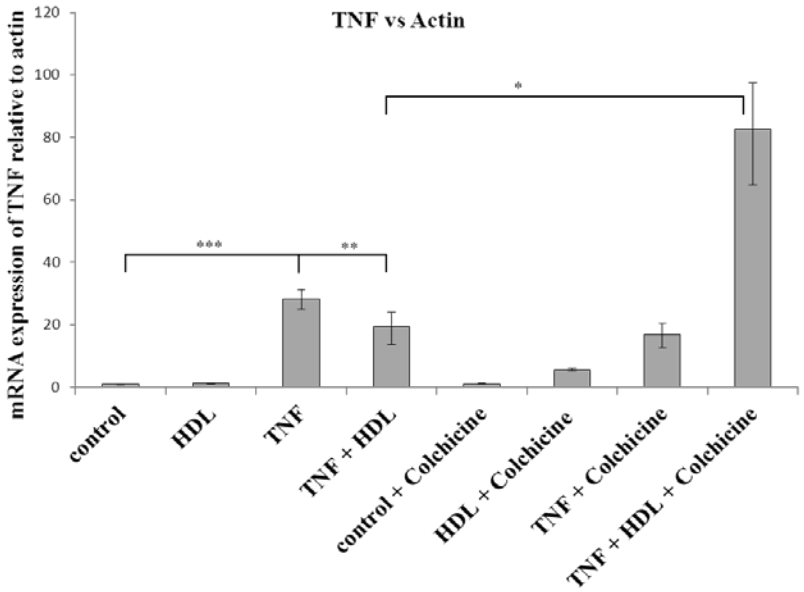


Figure 28: Anti-inflammatory effect of HDL is dependent on tubulin

5 DISCUSSION

5.1 Association of single nucleotide polymorphisms of FXR in IBD

The complex pathophysiology of IBD still remains largely unelucidated, although multiple factors, both genetic and environmental, are clearly involved. SNPs and mutations within several genes have been proposed to be associated with the risk to develop IBD.

In this report, we describe the identification of single nucleotide polymorphisms associated with the diagnosis of IBD within the *NR1H4* gene, encoding the nuclear receptor for bile acids, FXR, in a well-sized European cohort. Five *NR1H4* SNPs were analyzed, all of which have previously been studied in the context of other human disease conditions: *rs3863377*, *rs7138843*, *rs56163822*, *rs35724*, and *rs10860603*. The *NR1H4* variants *rs7138843* and *rs56163822* have been previously shown to be inversely associated with cholelithiasis in a Mexican population and may thus play a protective role in gallstone disease, while the variant *rs3863377* showed no association with cholelithiasis [204]. The variant *rs56163822* was found to be more common in a British control group than in patients with intrahepatic cholestasis of pregnancy (ICP), although this difference did not reach statistical significance [205]. *NR1H4* variants *rs35724* and *rs10860603* have been previously shown to be significantly associated with elevated body mass index and obesity [206]. IBD is often associated with hepatobiliary manifestations [207, 208], implying that the etiology of the diseases affecting the two organs, intestine and liver, may have common factors, also supported by our findings that the same *NR1H4* genetic variants may be associated with both. The variant *rs3863377* is located in the 5' region of the *NR1H4* gene, whereas the *rs7138843* lies within the *NR1H4* intron 7 and variants *rs35724* and *rs10860603* within *NR1H4* intron 9. In none of these cases is it known, how the presence of the SNP may affect the expression and/or molecular function of FXR. As the SNP *rs3863377* is located within the 5' region, it may alter a binding site for a transcription factor and may thus affect *NR1H4* gene expression. The intronic SNPs *rs7138843*, *rs10860603*, and *rs35724* could potentially influence splicing of the FXR mRNA. The substitution *-1G>T* in *rs56163822* lies in the base position adjacent to the translation initiation site, and was shown to lead to reduced FXR protein expression and decreased

level of FXR-dependent promoter activation in human embryonic kidney cells. In another study the functional activity of the *-1G>T* variant also appeared to be compromised, although transcriptional and translational efficiencies of the variant appeared comparable to the wild-type in cell-free assays and in HeLa cells [209]. Interestingly, the mRNA expression levels of the FXR target genes *SHP* and *OATP1B3* are significantly reduced in the livers of the carriers of the *rs56163822* allele, while the FXR mRNA expression level remains comparable, further indicating that this polymorphism may rather lead to weakened function than to reduced expression level of FXR.

In our current genotyping analysis we have found that for the *NR1H4* variant *rs3863377*, the IBD population has a significantly lower frequency of carriers of the rarer allele than the healthy population, suggesting that this 5' region SNP may confer a protective effect against the disease. In the case of the *rs56163822 NR1H4* variant, the rare allele is significantly more prevalent in the IBD population, suggesting that previously reported reduced FXR function exhibited by this variant may contribute to IBD pathogenesis. In the case of the rare *NR1H4* variant under study, *rs7138843*, and the common SNPs *rs10860603* and *rs35724*, no significant differences between the study populations were observed. In agreement with the associations observed for two of the five single SNP variants, the predicted global haplotype pattern was significantly different in IBD patients and non-IBD controls.

In our study, five *NR1H4* SNPs were investigated. During the preparation of our manuscript, Nijmeijer *et al* [210] published a study showing that mRNA expression of FXR and its target gene *SHP* are decreased in the ileum of Crohn's disease patients, in further support of the importance of the role for FXR in IBD. These authors also studied potential association of nine *NR1H4* SNPs with IBD in a Dutch population, but did not discover any associations that remained significant upon correction for multiple testing. We note that in their analysis Nijmeijer *et al* did not include the SNP *rs3863377*, the inverse association by which with IBD remained significant even after Bonferroni correction in the current study. As numerous further polymorphisms are known to exist in the *NR1H4* gene (www.ncbi.nlm.nih.gov/snp/), our report, as well as that by Nijmeijer *et al*, serve as initial characterizations of the role of FXR genetic variants in IBD. Furthermore, these findings warrant further studies into genetic variants in the *NR1H4*

gene in the context of other inflammatory conditions affecting further tissues that express FXR. FXR ligands, such as the hydrophilic bile acid ursodeoxycholic acid, have been proposed as attractive options for the therapy of liver diseases, such as cholestatic disease and non-alcoholic fatty liver disease [211]. Our finding that FXR genetic variants are associated with IBD, together with prior observations on FXR expression being altered in Crohn's disease [210] and on FXR promoting intestinal barrier integrity [27] and antibacterial defence [26], further emphasizes the potential benefits of FXR ligand administration also in IBD. We further speculate that testing for genetic variation in the *NR1H4* gene may contribute to the early IBD diagnosis and prediction of therapy response in the future.

5.2 HDL and its major protein ApoA1 suppress intestinal inflammation

IBD patients of both genders display dyslipidemia with significantly lower HDL-cholesterol (C). Since HDL is a negative acute phase reactant and since the intestine is the second most important site of HDL production, the low HDL cholesterol has traditionally been interpreted to be the consequence of IBD. Since HDL exerts many anti-inflammatory activities on many cells including endothelial cells, pancreatic beta cells and leukocytes, we hypothesized that HDL may also modulate intestinal inflammation [7, 212-215]. Both *in vitro* and *in vivo* we found evidence for anti-inflammatory effects of HDL and ApoA1 in the intestine.

The pro-inflammatory cytokine TNF is a key mediatior of mucosal inflammation in both Crohn's disease (CD) and ulcerative colitis (UC) and alters the integrity of epithelial and endothelial cell barriers [216, 217]. Increased concentrations of TNF have been found in blood, mucosa and faeces of CD patients. In several animal models of IBD, genetic ablation or anti-TNF treatment result in amelioration of mucosal inflammation. Furthermore, infliximab, a monoclonal antibody against TNF has been effective in clinical management of IBD [92, 150, 218, 219]. We therefore used TNF as a pro-inflammatory stimulator for our *in vitro* experiments. Preincubation of coloncarcinoma T84 cells with HDL or ApoA1 for 18 hrs significantly suppressed TNF triggered mRNA expression of IL-8, ICAM and TNF by inhibiting NF-κB promoter activity as measured by relative

luciferase activity and NF-κB DNA binding activity by EMSA. These findings are very similar to those previously made in experiments with endothelial cells and vascular smooth muscle cells where HDL was also found to reduce cytokine production and adhesion molecule expression via inhibition of NF-κB [143, 220, 221].

Importantly the anti-inflammatory properties of HDL and ApoA1 could be reproduced in an animal model of colitis. Upon DSS-treatment, ApoA1 KO phenotype exhibited a more severe intestinal inflammation as compared to WT mice as indicated by increased mucosal damage upon both colonoscopy and histology, shorter colon length, as well as increased intestinal MPO activity and mRNA expression of TNF and ICAM. Conversely, ApoA1 Tg mice, which have elevated levels of HDL, were partially protected from DSS induced mucosal damage as indicated by less severe symptoms upon colonoscopy and less severe increases in MPO activity. The functional importance of the attenuated intestinal inflammation in ApoA1 Tg mice is highlighted by the fact that WT mice and ApoA1 KO mice but not ApoA1 Tg mice, lost body weight upon DSS treatment. These observations are in very good agreement with previous findings of Cuzzocrea and colleagues who showed that administration of reconstituted HDL reduces intestinal inflammation in animals with splanchnic artery occlusion shock or dinitrobenzene sulfonic acid (DNBS)-induced colitis [222].

HDL and ApoA1 have been increasingly recognized as a part of innate immune system for their ability to bind and neutralize the toxic effects of lipopolysaccharide (LPS), a bacterial product that can activate Toll-like receptors stimulating the secretion of cytokines [223]. This function may be of special importance in the control of barrier integrity, for example in the vascular endothelium and the intestinal epithelium.

5.3 Anti-inflammatory effect of HDL and ApoA1 is mediated via activation of autophagic pathway

In this study, we report that HDL and ApoA1 induce autophagy in T84 cells. We provide evidence that anti-inflammatory effects exerted by HDL on T84 cells could be mediated via activation of autophagy. We also show that inhibition of autophagy reverse the anti-inflammatory effect exerted by HDL on T84 cells. Increase in LC3-II is an indicator of

pre-autophagosome formation. In our experiments, we observed increased processing of LC3 in the presence of HDL and ApoA1. This correlated with a decrease in phosphorylation of mTOR, a negative regulator of autophagy. Chemical inhibition of autophagic pathway using Wortmannin and 3-Methyl adenine decreased the ability of HDL to decrease TNF induced NF- κB responsive promoter activity as well as expression of TNF mRNA expression. Interestingly pretreatment of T84 cells with Bafilomycin A1 which inhibits the late stages of autophagosome fusion to lysosomes also inhibited the suppressive effect mediated by HDL on TNF induced mRNA expression of TNF. The intracellular transport of HDL seems to be tubulin mediated as demonstrated by the reverse effect of HDL when T84 cells were incubated with Colchicine, a tubulin blocker.

Where as SiRNA knockdown of LC3 significantly decreased the ability of HDL to suppress inflammation, SiRNA knock down of HDL receptors SRB1 and ABCA1 did not have any effect (data not shown). This observation co-ordinated well with our studies in human fibroblasts harboring ATG16L1 mutation. Further, we observed uptake of gold labeled HDL in autophagosomes which clearly depicted engulfed organelles such as mitochondria and these were found in close proximity to lipid droplets.

These results give an initial indication that autophagy might regulate inflammatory pathways in intestinal cells and HDL might modulate inflammation via autophagy. Genetic variation and dysfunction in autophagy genes IRGM and ATG16L1 has been implicated in improper bacterial handling and abnormalities in Paneth cells [160]. Recently Scharl *et.al* published an interesting study showing that PTPN2 maintain intestinal barrier functions and limits the effects of proinflammatory cytokines by up-regulating autophagy [224]. This study also shows that expression of autophagy proteins ATG16L1, IRGM, LC3 and ATG5-ATG12 are significantly decreased in inflamed mucosa of CD patients. In human endothelial cells, HDL has been shown to inhibit Oxidised LDL induced ER stress and autophagy [225]. Our study therefore depicts a tissue specific function of HDL, taking into account that intestine plays a major role besides liver in HDL/ApoA1 synthesis and metabolism and the high antigenic exposure in intestine bring together, a relevant role of HDL in maintaining barrier integrity of intestine by modulating autophagy. Possibly HDL recruit specific substrates such as phosphorylated IKK for autophagy mediated degradation there by suppressing inflammation.

CONCLUSIONS/ FUTURE PERSPECTIVES

In conclusion, the results of first project support the role for FXR as a modulator of intestinal inflammation and as an important player in enteroprotection. The link between the bile acid receptor FXR and IBD also further emphasizes the potential importance of bile acid homeostasis and metabolism in the pathogenesis of IBD. The experiments presented in project 2 demonstrate the capability of HDL and ApoA1 in protecting intestinal cells against inflammation induced damage. We show that HDL and ApoA1 suppress intestinal inflammation by attenuating NF-κB signalling. Present day therapy of IBD consists of salicylates, corticosteroids and immunosuppressants, most of which cause severe side effects. Modulation of HDL or ApoA1 serum levels may be an attractive approach to treat the chronic inflammatory reaction in IBD patients. In the third project, we provide evidence that HDL might modulate autophagy and thereby mediate suppression of intestinal inflammation. However there are several limitations to the present study. Despite the progress outlined above, there remain important questions that require further study in relation to a more complete understanding of mechanisms by which FXR, HDL and ApoA1 modulate inflammation. These include:

- Functional analysis of FXR variants, *rs3863377 and rs56163822* which associated with IBD, could give more insight into how exactly the variants influence FXR gene expression and function and its significance to IBD.
- The *in vivo* data demonstrating anti-inflammatory effect of ApoA1 was done in an acute DSS colitis model. It is important to reproduce these result in a chronic model of DSS colitis which resemble the chronic phase of UC and CD and enable us to elucidate the significance of ApoA1 in chronic inflammation.
- It is also important to put some thoughts into the mechanisms upstream of autophagy which HDL might modulate. Presently we demonstrate that HDL is take up by the process of autophagy and induce genes relevant for autophagy. We also show that lack of autophagy genes decreases the anti-inflammatory effect of HDL, but how exactly HDL induced autophagy suppresses inflammation is still a question to resolve.

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DECLARATION

- The haplotype analysis of FXR variants (Tables 4, 4.1 and 4.2) as well as Figure 7 was performed by Jessica Mwinyi
- In Animal experiments, mice were sacrificed by Kerstin Atrott, Colonoscopy was performed by Anne Terhalle.
- Scores of Colonoscopy and Histology was blinded by Alexandra Cee and Anne Terhalle respectively
- Electron micrographs were generated at Centre for microscopy and image analysis (UZH) by Bruno Gühl and Gery Barmettler

CURRICULUM VITAE

Personal Data

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Education

2009-2012

PhD research studies in the group of Prof. Dr. Dr. Gerhard Rogler (Division of Gastroenterology and Hepatology, University Hospital Zürich)

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Topic: Role of bile acid receptor FXR and high density lipoprotein (Hdl) in inflammatory bowel disease (IBD)

2007 – 2009

Masters degree Biochemistry

(University of Zürich, Switzerland)

Master thesis in the group of Prof. Ari Helenius

Topic: Characterization of interaction of vaccinia viral proteins containing f-box and btb-domain with cellular ubiquitin ligase complex.

Feb 2008- April 2008

Semester work in the group of Prof. Benjamin Schuler at University Zürich.

Topic: Determination of the folding pattern of a HIV mutant protein domain Integrase 45 using single molecule fluorescence spectroscopy.

April 2007 – July 2007

Practical at the cancer institute, University Zürich

Topic: Construction of viral expression vectors employed to study the regulation of Hypoxia Inducible Factor (HIF-1α) in cell lines.

2003 – 2007
Diploma chemical engineering
(Fachhochschule Nordwestschweiz (FHNW), Muttenz, Switzerland)
Diploma work at the University of Palermo under Prof. Dr. Annamaria Puglia and Prof. Daniel Gygax (FHBB, Muttenz)

Topic: Proteomic analysis to reveal the primary metabolic enzymes involved in antibiotic production in Amycolatopsis balhimycina using 2D-PAGE analysis.

2001-1997
Predegree
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TEACHING EXPERIENCE

2009-2012 Teaching assistant at practical courses.for 1st and 2nd year medical students Institute of Physiology, University of Zurich

Topics: Membrane transport, Heart, Blood, Signal transduction

Language Skills

English	Fluent spoken and written
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International Experience

September 2011

Presenter at the Post graduate symposium (University of Queensland, Brisbane), conducted by the Swiss Embassy

April 2011

Presenter at the Digestive disease week (DDW) held in Chicago

September 2006 – Feb 2007

Diploma work at the University of Palermo under Prof.Dr. Annamaria Puglia and Prof. Dr. Daniel Gygax (FHNW, Muttenz)

Publications

1. Attinkara R, Mwinyi J, Truninger K, Regula J, Gaj P, Rogler G, Kullak-Ublick GA, Eloranta JJ., Association of genetic variation in the NR1H4 gene, encoding the nuclear bile acid receptor FXR, with inflammatory bowel disease, ***BMC Res Notes***, 2012 Aug 28;5(1):461